PHYTOPATHOLOGY

VOLUME 26 NUMBER 6

JUNE, 1936

VIRUS CONCENTRATION IN RELATION TO ACQUIRED IMMUNITY FROM TOBACCO RING SPOT[1]

W. C. PRICE

(Accepted for publication February 26, 1936)

INTRODUCTION

In 1932, the writer (5) described recovery of several species of Nicotiana from tobacco ring spot. It was shown that this recovery is a normal behavior of plants infected with the disease, that it occurs under most environmental conditions, and that it is distinguished from masking of symptoms, which occurs only under exceptional environmental conditions. It was, furthermore, shown that recovered plants cannot be induced to develop symptoms by reinoculation with virus from diseased plants. This phenomenon of recovery and subsequent failure to develop symptoms on reinoculation was referred to as acquired immunity. It differs from acquired immunity from most animal diseases in that it is not accompanied by complete disappearance of virus. Because of the retention of virus by recovered plants, several workers, notably Smith (7), Köhler (4), and Valleau (10), have objected to the use of the term acquired immunity in this connection. Smith prefers to consider the phenomenon as acquired immunity from the clinical picture of ring spot or as acquired tolerance. Köhler, likewise, prefers the term acquired tolerance. Valleau, on the other hand, maintains that the so-called recovery is nothing more than a stage of the disease; that the plants do not actually recover from the disease, but merely develop patternless leaves in which the cells are parasitized to the limit.

In order to obtain a better understanding of the nature of this phenomenon, a study was made of the relative virus concentration in various parts of recovered and diseased tobacco plants. The results furnish additional evidence that here, indeed, is a non-sterile immunity comparable with that that obtains in a number of virus diseases of animals.

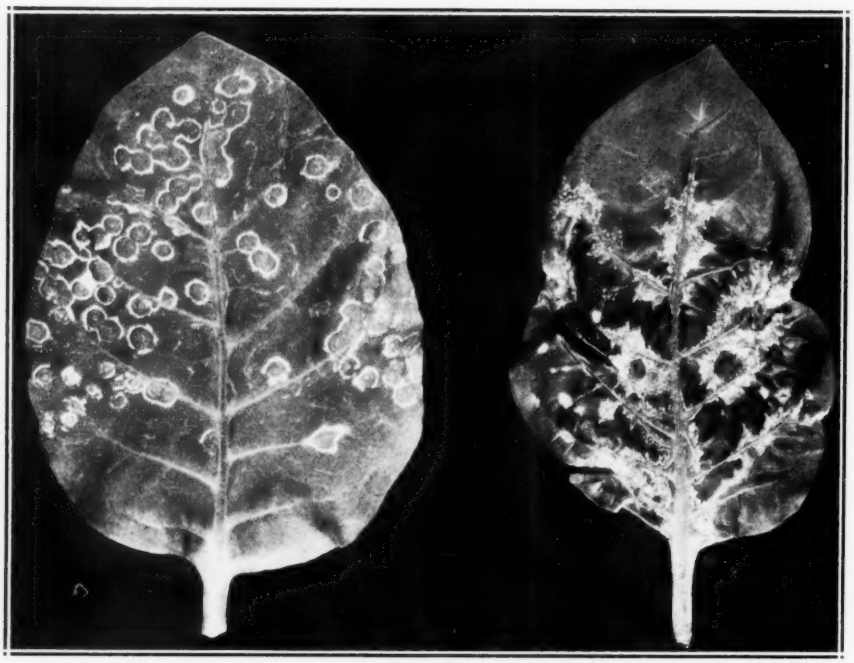
DESCRIPTION OF THE DISEASE

A detailed description of tobacco ring spot has been given by Wingard (11) and by Price (5). Only a brief review of the characteristic symptoms

[1] Published at the expense of The Rockefeller Institute for Medical Research, Princeton, N. J., out of the order determined by the date of receipt of the manuscript. This practice in nowise delays the publication of manuscripts printed at the expense of The American Phytopathological Society or other agency.

produced in tobacco (*Nicotiana tabacum* L. var. Turkish) as different stages of the disease develop will be presented.

Transmission of the disease is readily obtained by the rubbing method of inoculation. Primary lesions develop in the inoculated leaves within 3 or 4 days. They consist of rings or circles of necrotic or chlorotic tissue alternating with rings of healthy-appearing tissue. They also may occur as solid necrotic spots. Figure 1 illustrates a leaf bearing necrotic primary lesions and a leaf showing systemic lesions.



Photographed by J. A. Carlile

FIG. 1. Leaves of Turkish tobacco showing (left) primary and (right) systemic necrotic lesions produced by ring-spot virus.

Systemic lesions appear 6 or more days after inoculation. They are similar to the primary lesions, but usually are larger, especially when centered on large veins. They are also fewer in number than the primary lesions produced by rubbing with undiluted inoculum.

The third stage in the disease, and one that marks the beginning of the plant's recovery, is initiated from 10 to 14 days after inoculation. Leaves are produced that show wavy lines of chlorotic or necrotic tissue extending across their apical portions, leaving the basal portions free from necrosis and healthy in appearance (Fig. 2). The first of such leaves to develop have

Photographed by J. A. Carlile

FIG. 2. Leaves of Turkish tobacco showing the characteristic oak-leaf pattern that occurs during the process of recovery from tobacco ring spot. Such leaves are referred to as partly recovered leaves, since symptoms appear only on their apical portions.

only a small fraction of the base free from necrosis but, as new leaves continue to appear, the lines are situated closer and closer to the tips of the leaves leaving a larger and larger fraction of the basal part free of symptoms.

Finally, leaves are produced that are completely free from chlorotic and necrotic lesions and are, as a rule, indistinguishable from healthy leaves. The leaves produced after this fourth stage has been reached never develop lesions characteristic of the onset of the disease.

Figure 3 shows a photograph of a plant that has recovered from ring spot. It will be seen from an examination of this figure that the leaves near the tip of the plant are free from lesions and healthy in appearance and that leaves below the tip show characteristic ring-spot lesions.

For convenience, the healthy-appearing leaves on plants that have recovered from ring spot will be referred to as recovered leaves, and those bearing necrotic lesions as diseased leaves.

MATERIALS AND METHODS

Ring-spot Virus: A brief description of a disease later shown to be tobacco ring spot was given by Valleau[2] in 1921. A more detailed description was given by Fromme and Wingard (2, p. 16–17) in 1922. It was not, however,

[2] Valleau, [W. D.]. Kentucky, a disease of tobacco. U. S. Dept. Agr. Bur. Plant Indus. Plant Disease Bull. 5: 107. 1921. [Mimeographed]

Photographed by J. A. Carlile

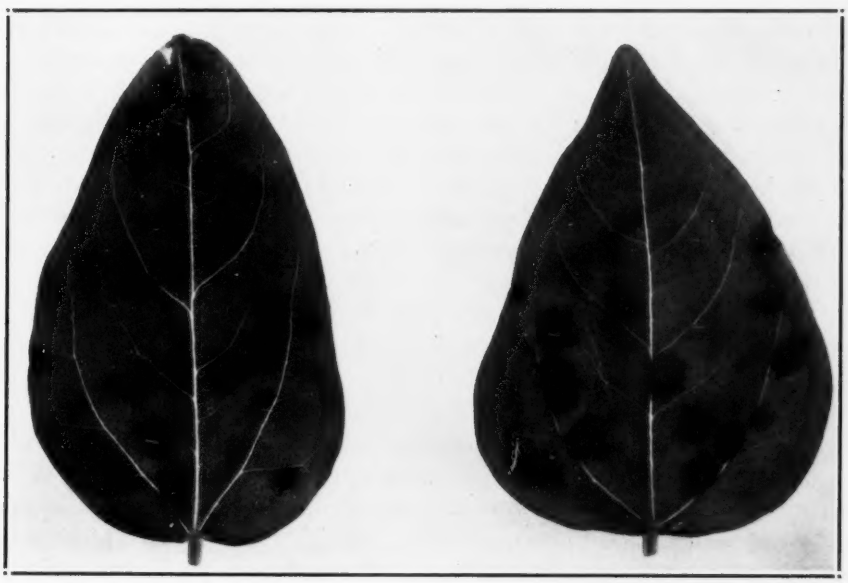
FIG. 3. A Turkish tobacco plant infected with ring spot (left) and a healthy plant of the same age (right). The infected plant has recovered from the disease and developed a number of healthy-appearing leaves at the tip. Symptoms appear only on the basal leaves, which were infected during the onset of the disease.

until the publication of a paper by Fromme, Wingard and Priode (3) in 1927 that the disease was definitely shown to be infectious, although Fromme[3] reported in 1923 that Valleau had obtained 2 infections out of about 200 attempts by the rubbing method. The writer obtained the virus from Wingard in 1929 and has maintained it continuously in tobacco plants since that time. The virus does not appear to be identical with either the green or yellow ring-spot viruses described by Valleau (9), but it is likely that it may be a strain of his green ring-spot virus.

Host Plants. *Nicotiana tabacum* L. var. Turkish served as the host plant in all the studies to be reported in this paper. The plants were grown in 4- or 6-inch porous clay pots in a greenhouse. They were, as a rule, inoculated when from 2 to 4 inches in height. Inoculations were invariably made by means of the rubbing method.

[3] Fromme, [F. D.]. Ring spot or hieroglyphics. U. S. Dept. Agr. Bur. Plant Indus. Plant Disease Bull. Sup. **26**: 142. 1923. [Mimeographed.]

Preparation of Virus Samples for Inoculation. In determining the virus concentration in different plants or in different leaves on a plant, the preparation of the virus sample was as follows: A 2-inch square of tissue was cut from the leaf to be tested, was thoroughly macerated in a sterile mortar, and was diluted with 4 cc. of water. The solution thus obtained was applied to leaves of the test plants by means of a small square of cheese cloth. In those cases in which two or more samples were taken from the same leaf, a $1\frac{1}{2}$-inch square of tissue was used. For measurements made using stems as a source of virus, a section of stem, $1\frac{1}{2}$ to 2 inches in length, was cut from a point near the tip of the plant tested. Measurements of virus concentration in roots were made in the same manner, an approximately equal volume of root tissue being used for each test. The actual juice dilution of virus used is estimated to be approximately 1:5 to 1:10 for the leaf samples and 1:1.5 to 1:2 for the stem and root samples.



Photographed by J. A. Carlile

FIG. 4. Necrotic primary lesions produced by tobacco ring-spot virus in leaves of Black cowpea. The photograph was taken 5 days after inoculation.

Measurement of Virus Concentration. An estimate of the virus concentration in the different samples tested was secured by inoculating leaves of the Black variety of cowpea, *Vigna sinensis* Endl., and counting the numbers of lesions produced. It was found previously (5) that the numbers of lesions produced in the cowpea leaves decreased progressively as the virus content of the inoculum decreased. Figure 4 illustrates the lesions pro-

duced in this host plant 5 days after inoculation. The plan was to inoculate 40 cowpea leaves with each of the virus samples. Accordingly, the cowpeas were planted 6 to a pot, 4-inch pots being used in each instance. Five pots of cowpeas were used for each of the virus samples tested. Not all the seed germinated and grew, the numbers varying from 2 to 12 leaves (1 to 6 plants) per pot available for test. The plants were inoculated when they were from 6 to 9 days old, only the first pair of true leaves on each plant being used. These leaves had attained a size of from $\frac{1}{2}$ to $\frac{3}{4}$ their maximum at the time of inoculation. The figures given in the tables represent the number of lesions obtained for 40 cowpea leaves with each of the virus samples tested. The figures were obtained by dividing the actual number of lesions by the total number of leaves and multiplying the quotient by 40. This was done in order to eliminate fractions, and because 40 was the actual number of leaves used in many instances.

Statistical Analysis.[4] The data obtained from quantitative measurements of ring-spot virus were subjected to the analysis of variance. The analysis consists essentially in the segregation of that part of the variation that is associated with specific causes and provides an estimate of the experimental error freed from the variability of known origin. A discussion of the analysis of variance is to be found in Fisher's text (1). It has been applied recently to data from plant-virus studies by Youden and Beale (12). In the present paper the values designated as significant, and those designated as highly significant, were determined by the application of Snedecor's (8) table XXXV. The table was computed by Snedecor from Fisher's table VI. The values designated as significant correspond to Fisher's 5 per cent point (odds of 20 to 1), while those designated as highly significant correspond to the 1 per cent (odds of 100 to 1).

At the outset it may be said that there are certain definite sources of variation to be taken into consideration in interpreting the data presented in the following pages. The experiments were, as a rule, designed to determine the difference in concentration between virus taken from different plants or from different parts of plants. Variation arising from this source usually accounts for a large fraction of the total. Of the other sources of variation common to all the experiments, that between tests is perhaps the most consistently significant throughout. The large value attributed to this variance emphasizes the importance of making comparisons only between measurements made at the same time and under the same environmental conditions. Probably the difference in susceptibility of cowpea plants grown under slightly different environmental conditions accounts for a large part of the variation between tests. In most cases the interaction has proved to

[4] The writer is greatly indebted to Dr. John W. Gowen for advice and assistance in the statistical analysis of the data presented in this paper.

be significant, indicating a rather large variation in trend between the different tests in the same experiment. Another source of variation is the origin of the virus samples. The large mean square for the variation between inoculum samples in the same experiment-and-treatment subclass shows that, in nearly all cases, virus samples taken from comparable groups of plants grown under similar conditions varied considerably in concentration. It is likely that plants grown under different environmental conditions would vary even more widely in virus content. The other sources of variation to be considered are concerned with the susceptibility of cowpea plants to ring-spot virus. With one exception, plants in different pots showed a greater variation than plants in the same pot. Likewise, leaves on the same plants varied less than leaves on different plants.

EXPERIMENTAL

I. Multiplication of Virus in Recovered Plants

While this paper is concerned chiefly with virus concentration, it should not be out of place to refer here briefly to multiplication, or increase, of virus in plants that have recovered from ring spot, inasmuch as this question may have a direct bearing upon some phases of the main problem. It was found earlier (5) that recovered plants retained the virus of this disease when grown through 3 generations from cuttings. This finding was not, however, considered as conclusive evidence that the virus multiplies in recovered plants, since the treatment did not serve to dilute the virus originally present in the plant to a great enough extent. In this work the plan was to grow recovered plants through a sufficient number of generations from cuttings to insure a high dilution of the virus originally present, and then to test these plants for presence of virus. Twenty Turkish tobacco plants were inoculated with ring-spot virus and allowed to recover from the disease. When the recovered plants had attained a height of from 2 to 3 feet, tip cuttings were taken from each, rooted in a mixture of peat moss and sand, and, finally, potted in 6-inch pots. A second set of cuttings was later taken from each of the plants grown from cuttings of the first set. The process was continued until a total of 10 generations from cuttings had been grown over a period of a little more than 2 years. Occasionally, to prevent the young plants from going to seed, it was necessary to cut off the tops and allow the axillary buds to develop. Assuming an equal distribution of virus in each plant, it is estimated that, averaged on the basis of green weight, approximately 1/16 of the virus present in the plant was retained in the cutting. Over the period covered by the experiment, the virus originally present should have attained a dilution of more than 1×10^{-12} in the 10th series of cuttings. At no time during the 2 years did necrotic or chlorotic lesions appear in the plants. They occasionally showed an inconspicuous

yellowing or necrosis along the leaf edges similar to that previously illustrated and described (5), but, as a rule, they showed neither chlorosis nor necrosis. They were, however, somewhat more stunted than plants grown from seed. Each of the 20 plants grown from cuttings was tested for ring-spot virus by inoculation of healthy Turkish tobacco plants. An appreciable amount of virus was found to be present in each of the 20 plants tested. Since infection is not ordinarily obtained in tobacco with dilutions greater than 1×10^{-4}, it is concluded that the virus must have multiplied in the recovered plants. The symptoms produced in tobacco by virus from recovered plants grown through 10 generations from cuttings were no different from those produced by virus from diseased plants. The virus was, therefore, not attenuated after 2 years in recovered plants.

II. Virus Concentration of Leaves of Recovered and Diseased Plants

An experiment was performed for the purpose of determining whether or not the virus concentration of leaves of recovered plants differs significantly from that of leaves of diseased plants. Measurements were made of virus in 10 plants that had recovered from ring spot and in 10 plants showing acute symptoms of the disease. A leaf showing numerous necrotic lesions was selected from each of the diseased plants and used as a source of inoculum. A leaf of approximately the same size and, as nearly as possible, of the same physiological age was selected from each recovered plant and likewise used as a source of inoculum. Each sample was used to inoculate 5 pots of cowpeas. The inoculations were alternated so that the first set of 5 pots was inoculated with juice from a recovered plant and the next set with juice

TABLE 1.—*Relative virus concentration of leaves from Recovered (R) and diseased (D) tobacco plants infected with ring spot as indicated by the numbers of lesions produced in 40 cowpea leaves*

Plant No.	Test No. 1		Test No. 2		Test No. 3		Test No. 4	
	R	D	R	D	R	D	R	D
1	79	484	50	295	61	83	15	283
2	45	521	17	209	25	174	21	130
3	81	555	114	410	91	107	62	237
4	161	592	74	244	31	264	34	89
5	140	387	60	386	8	46	31	81
6	50	353	50	325	19	71	43	193
7	49	430	34	688	34	123	29	232
8	118	254	82	201	142	46	67	309
9	27	650	27	232	72	227	30	283
10	39	527	42	173	9	44	16	126
Total	789	4753	550	3163	492	1185	348	1693

from a diseased plant. The experiment was repeated on 3 different occasions with 3 different sets of plants. The average numbers of lesions obtained in the 4 tests are given in table 1. The results show that the diseased leaves contained more than 5 times as much virus as recovered leaves.

The data of table 1 were subjected to the analysis of variance, the results of the analysis being shown in table 2. In this experiment, 40 cowpea leaves

TABLE 2.—*Statistical analysis of the data summarized in table 1*

Sources of variation	Degrees of freedom	Sum of squares	Mean square
Between leaves on same plants	1,600	10,797.5	6.7
Between plants in same pot	1,200	24,891.4	20.7
Between pots of same inoculum	320	10,325.6	32.3
Between inoculum samples in same test-and-treatment subclass	72	12,378.2	171.9
Between test-and-treatment subclass	7	43,048.1	6,149.7

were used for each recovered or diseased plant; 400 leaves were inoculated with virus from recovered plants and 400 with virus from diseased plants in each of the 4 tests. A total of 3200 cowpea leaves were, therefore, used. While 5 pots of cowpeas were used for each recovered or diseased plant, the number of leaves per pot, unfortunately, varied from 2 to 12 with a harmonic mean of 7.27. The unequal number of leaves per pot introduced some heterogeneity in the direct analysis of the 3 sources of variation in the between-test-and-treatment subclass; between the different tests, between virus derived from recovered or diseased plants, and for the differential effects of the virus. To obviate this difficulty, the mean numbers of lesions per leaf for each pot were used and table 3 was derived.

TABLE 3.—*Analysis of the 3 sources of variation in the between-test-and-treatment subclass of table 2*

Sources of variation	Degrees of freedom	Sum of squares	Mean square
Between tests	3	1,305.6	435.2
Between virus from recovered *vs.* diseased leaves	1	2,930.1	2,930.1
Interaction	3	883.9	294.6

The appropriate variance to test the significance of the items in table 3 is that between pots of the same inoculum, 32.3, divided by the harmonic mean of the plants per pot, 7.27; or perhaps a better means of comparison is to multiply the mean squares of table 3 by 7.27 and then compare them

with those of table 2. These values are: between tests, 3163.9; between virus of recovered and diseased plants, 21,301.8; and interaction, 2141.7.

The data show that the factor in the variation of the lesions per leaf within the experiment that contributed most to the total variation was the source of the virus, whether it was obtained from a plant that had recovered or from one that was diseased. The statistical significance of the result is beyond question, since the mean square due to this cause, 21,301.8, divided by the estimated variance, 32.3, is 659.5, where 6.7 would be highly significant.

The variation due to differences in the conditions under which the 4 tests were performed has likewise resulted in a rather large effect. The significant value of the interaction indicates that the relative virulence of the recovered and diseased virus samples varied from test to test. Turning to table 2, the significantly large mean square of 171.9 for the inoculum samples in the same test-and-treatment subclass shows that the tobacco leaves varied in virus concentration.

The 2 mean squares—between leaves of the same plant and between plants in the same pot—bear on two problems of somewhat secondary importance to this experiment but of primary interest in connection with the technique of such experiments and the general factors affecting virus diseases. The small mean square between the lesions of the two leaves of the same plant treated in the same manner shows the close similarity in the reactions of these leaves to the same stimulus. In view of this fact, a better technique than that used for the experiment would have been to treat one of the leaves of the plant with the virus of the recovered plant and the other leaf with the virus of the diseased plant, and then make the comparison. In this connection it should be noted that Samuel and Bald (6) have suggested the use of the "half-leaf" method for estimation of tobacco-mosaic virus concentration and Youden and Beale (12) have suggested a Latin-square arrangement of leaves of test plants in order to minimize the variation resulting from differences between test plants.

The virus reactions on the different plants vary, showing that there is a factor innate in the test cowpea plants that tends to make them more or less resistant to the virus. These facts are evident in all subsequent experiments.

III. Virus Concentration of Healthy-appearing and Diseased Parts of Partly Recovered Leaves

In the process of recovery of a tobacco plant from the ring-spot disease, leaves are produced that develop symptoms only on their tips, as has already been mentioned. Such leaves usually show an oak-leaf pattern, consisting of one or more zigzag lines of necrotic tissue, near the middle of the leaf, and a number of zonate circular lesions near the tip. Such a leaf is illustrated in

figure 2. In this paper, the basal part of a leaf showing an oak-leaf pattern will, for convenience, be referred to as the recovered portion, the apical part as the diseased portion, and the entire leaf as a partly-recovered leaf. Two tests were conducted, in each of which measurements were made of the virus concentration in apical and basal parts of partly-recovered tobacco leaves. The results of the tests are given in table 4. The statistical analysis of the experiment is summarized in table 5. The data show that the diseased parts

TABLE 4.—*Relative virus concentration of recovered (R) and diseased (D) parts of partly recovered leaves as indicated by the average number of lesions per leaf multiplied by 40*[a]

Plant No.	Test No. 1		Test No. 2	
	R	D	R	D
1	161	520	325	464
2	262	668	201	289
3	299	425	188	224
4	147	359	129	166
5	124	276	68	90
6	208	438	236	203
7	163	289	90	155
8	114	145	117	240
9	56	205	62	192
10	64	126	120	318
Total	1598	3451	1536	2341

[a] The average was multiplied by 40 to avoid fractions and because in many instances the actual number of leaves used for test was 40.

TABLE 5.—*Statistical analysis of the data summarized in table 4*

Sources of variation	Degrees of freedom	Sum of squares	Mean square
Between leaves of same plants	899	10,225.5	11.4
Between plants of same pot	699	21,398.9	30.6
Between pots of same inoculum	160	9,716.2	60.7
Between inoculum samples in same test and treatment	36	12,985.7	360.7
Variance based on pot means and adjusted for the harmonic mean number of lesions per pot			
Between tests	1	837.8	837.8
Between virus from recovered *vs.* diseased parts of same leaves	1	4,440.2	4,440.2
Interaction	1	690.5	690.5

Harmonic mean number of leaves per pot is 8.61.

of the leaf contained considerably more virus than the recovered parts. The result is clearly significant, since the ratio of the mean squares, 4440.2/60.7 or 73.1, is much more than 3.9 for significance.

IV. Virus Concentration of Apical and Basal Parts of Recovered and of Diseased Leaves

As a check to the experiment discussed in Section III, measurements were made of the virus concentration in apical and basal parts of recovered leaves and, likewise, in apical and basal parts of diseased leaves. Ten recovered and 10 diseased leaves were used for the experiment. The diseased and recovered leaves were tested at different times. The results of this experiment are given in table 6 and an analysis of the results in table 7. The data

TABLE 6.—*Relative virus concentration of apical (A) and basal (B) parts of diseased and recovered leaves as indicated by the average numbers of lesions per leaf multiplied by 40*[a]

Plant No.	Diseased leaves		Recovered leaves	
	A	B	A	B
1	849	818	326	285
2	668	721	54	88
3	454	494	60	173
4	344	222	115	191
5	537	379	186	342
6	109	250	226	224
7	394	193	232	301
8	355	407	252	256
9	493	486	226	236
10	393	709	247	209
Total	4596	4679	1924	2325

[a] See footnote, table 4.

show that there was no significant difference in the virus concentration of the apical and basal parts of either the diseased or recovered leaves. As might be expected from table 1, the largest contribution to the variation is that between recovered and diseased leaves. The interaction is not significant. Referring back to Section III, the difference in virus concentration between recovered and diseased parts of partly recovered leaves cannot be ascribed to a positional effect, but must result from a dissimilarity of recovered and diseased tissues. Consequently, it may be concluded that the healthy-appearing tissues are associated with a low virus content and the necrotic tissues with a high virus content. These tissues are sharply delimited as regards both external appearance and virus concentration.

TABLE 7.—*Statistical analysis of the data summarized in table 6*

Sources of variation	Degrees of freedom	Sum of squares	Mean square
Between leaves of same plants	673	13,199.3	19.6
Between plants of same pot	471	55,739.6	118.1
Between pots of same inoculum	160	23,984.7	149.9
Between inoculum samples in same test and treatment	36	19,328.2	536.9
Variance based on pot means and adjusted for the harmonic mean number of leaves per pot			
Between diseased and recovered leaves	1	12,914.9	12,914.9
Between virus from apical *vs.* basal parts of leaf	1	4.8	4.8
Interaction	1	68.7	68.7

Harmonic mean number of leaves per pot is 5.97.

V. Virus Concentration of Partly Recovered and Fully Recovered Leaves

A comparison of tables 1 and 4 has indicated that, while the virus concentration of diseased parts of partly recovered leaves is comparable with that of diseased whole leaves, the recovered parts of partly recovered leaves might have a slightly higher concentration of virus than recovered whole leaves. Two tests, therefore, were conducted in which a comparison was made between recovered parts of 10 partly recovered leaves and 10 fully recovered leaves taken from the same plants. The data are presented in table 8. Table 9 gives the statistical analysis of these data. The results

TABLE 8.—*Relative virus concentration of partly recovered (P) and fully recovered (F) leaves as indicated by the average numbers of lesions per leaf multiplied by 40*[a]

Plant No.	Test No. 1		Test No. 2	
	P	F	P	F
1	204	290	431	304
2	75	244	278	117
3	204	526	231	300
4	114	210	33	147
5	138	280	42	42
6	199	265	18	87
7	123	274	74	154
8	250	290	52	104
9	77	248	76	77
10	155	315	51	87
Total	1539	2942	1286	1419

[a] See footnote, table 4.

TABLE 9.—*Statistical analysis of the data summarized in table 8*

Sources of variation	Degrees of freedom	Sum of squares	Mean square
Between leaves of same plants	832	6,861.5	8.2
Between plants in same pot	632	18,202.6	28.8
Between pots of same inoculum	160	6,755.9	42.2
Between inoculum samples in same test and treatment	36	9,141.0	253.9
Variance based on pot means and adjusted for the harmonic mean number of lesions per pot			
Between tests	1	1,682.3	1,682.3
Between virus from partly recovered leaves *vs.* fully recovered leaves	1	1,336.3	1,336.3
Interaction	1	887.2	887.2

Harmonic mean number of leaves in pot is 7.81.

show that, within the 2 tests, the recovered parts of partly recovered leaves contained significantly less virus than fully recovered leaves from the same plants, the ratio of the mean squares, 1336.3/42.2, is 31.7 where 3.9 would be significant. The variation in materials as shown by the rather wide differences in the lesions of the 2 tests and the differential changes in the reaction of the virus as shown by the interaction, were both markedly significant.

The results are in conflict with those roughly obtained by a comparison of tables 1 and 4. Possibly an explanation for the significant difference in virus concentration between partly recovered and fully recovered leaves lies in the fact that the recovered leaves were, in every case, younger (taken from nearer the tip of the plant) than the partly recovered leaves. Another possible explanation is, however, the phenomenon of over-compensation frequently observed in vital processes. The tissues that are to recover may overreact to the virus causing a greater inhibition of virus multiplication than is the equilibrium value for the recovered plant. This equilibrium value may be established later in the fully recovered leaves.

VI. Virus Concentration of Inoculated Leaves of Recovered Plants, of Noninoculated Leaves of Recovered Plants, and of Inoculated Leaves of Previously Healthy Plants

Since it has been shown that recovered leaves almost invariably contain a lower concentration of virus than diseased leaves, an experiment was conducted to determine whether or not the virus content of recovered leaves could be increased by inoculating them with ring-spot virus. Healthy plants and recovered plants of as nearly the same age, size, and appearance as was possible to find were selected. The recovered plants were divided into 2

groups. One group of recovered plants, and all the healthy controls were inoculated by rubbing several leaves with ring-spot virus. The other group of recovered plants was not inoculated. Five days after inoculation, measurements were made of the virus concentration in inoculated leaves of the healthy controls, in inoculated leaves of the first group of recovered plants, and in noninoculated leaves of the second group of recovered plants. The data from 2 tests are given in table 10, and the analysis of the data in table 11. The comparison between the mean square for the virus source and the experimental error shows a highly significant difference, 24,340.7/77.8, a ratio of 312.9 to 1, where 3.9 would be significant. From the table containing the original data, it is obvious that differences in the 3 different virus treatments do not contribute equally to the variation. The estimate of the variance, 77.8, may be used to test the significance of these differences. The standard deviation is equal to $\sqrt{77.8}$, or 8.82. The total number of lesions produced

TABLE 10.—*Relative virus concentration of leaves of recovered plants (R), of reinoculated recovered plants (IR), and of inoculated healthy plants (IH), as indicated by the average numbers of lesions per leaf multiplied by 40*[a]

Plant No.	Test No. 1			Test No. 2		
	R	IR	IH	R	IR	IH
1	27	87	720	67	103	250
2	57	211	460	143	165	396
3	77	79	511	162	120	817
4	16	52	461	38	131	778
5	18	44	756	317	256	1084
6	37	100	478	18	139	753
7	61	89	297	85	48	478
8	8	37	475	139	122	360
9	72	35	331	135	184	403
10	43	35	430	190	122	129
Total	416	769	4919	1294	1390	5448

[a] See footnote, table 4.

by juice from the recovered plants is 1675 on 756 leaves; by juice from reinoculated plants, 1947 on 700 leaves; and by juice from inoculated healthy plants, 9509 on 722 leaves. The mean lesions per leaf for each group are 2.22, 2.78, and 13.17. The standard errors of the differences of these results are $8.82\sqrt{\frac{1}{756}+\frac{1}{700}}=0.463$, $8.82\sqrt{\frac{1}{756}+\frac{1}{722}}=0.459$, and $8.82\sqrt{\frac{1}{700}+\frac{1}{722}}=0.468$, respectively. The differences between the groups are $2.78-2.22=0.56$, $13.17-2.22=10.95$, and $13.17-2.78=10.39$, leading to the values of 1.2 with 1454 degrees of freedom, 23.86 with 1476 degrees of freedom, and

TABLE 11.—*Statistical analysis of the data summarized in table 10*

Sources of variation	Degrees of freedom	Sum of squares	Mean square
Between leaves of same plants	1,089	14,345.5	13.2
Between plants of same pot	789	70,778.7	89.7
Between pots of same inoculum	240	18,670.1	77.8
Between inoculum samples in same test and treatment	54	27,688.0	512.7
Variance based on pot means and adjusted for the harmonic mean number of leaves per pot			
Between tests	1	1,301.2	1,301.3
Between virus from recovered, inoculated recovered, and inoculated healthy plants	2	48,681.4	24,340.7
Interaction	2	77.8	38.9

Harmonic mean number of leaves per pot is 6.49.

TABLE 12.—*Relative virus concentration of roots of recovered (R) and diseased (D) plants as indicated by the average numbers of lesions per leaf multiplied by 40*[a]

Plant No.	Test No. 1		Test No. 2	
	R	D	R	D
1	39	125	232	324
2	43	126	125	295
3	120	157	94	141
4	143	147	90	185
5	76	92	195	388
6	67	85	106	278
7	69	64	53	132
8	43	57	101	128
9	42	52	175	83
10	15	33	82	54
Total	657	938	1253	2008

[a] See footnote, table 4.

22.20 with 1420 degrees of freedom. The results consequently show that the only significant difference was that between the inoculated healthy controls and either the recovered or reinoculated recovered plants. The recovered and reinoculated recovered leaves did not differ significantly in virus content. No evidence was obtained that the virus concentration of recovered leaves may be increased by inoculating them heavily with ring-spot virus.

VII. Virus Concentration of Roots of Recovered and Diseased Plants

Since it was shown that leaves of recovered and diseased plants differ significantly in virus concentration, experiments were undertaken to test

other parts of the plants. Measurements were made on the virus concentration of roots of recovered and diseased tobacco plants. Root samples that were approximately equal in volume were selected for the tests. Unfortunately, it was necessary to use recovered plants that were about 2 weeks older than the diseased plants. The roots were washed in tap water and macerated in a mortar with 4 cc. of water. Results of 2 tests with roots of recovered and diseased plants are given in table 12. A statistical analysis of the data is given in table 13, which shows that there was a significant difference in the virus content of the roots from recovered and diseased plants. The ratio of the mean square, 645.9, to the experimental error, 16.6, is 38.9 where 3.9 would be significant. Roots of recovered plants contained, on the average, considerably less virus than roots of diseased plants. That the roots varied widely in virus concentration is shown by the ratio of the mean square between inoculum samples in same test and treatment to the experimental error, $125.4/16.6 = 7.6$. The most significant difference was, however, that between tests.

VIII. Virus Concentration of Stems of Recovered and Diseased Plants

An experiment was performed in order to determine whether or not stems of diseased plants have a higher virus concentration than stems of recovered plants. A section of stem was removed from the tip part of each of 10 recovered and 10 diseased Turkish tobacco plants. The recovered plants used for test were somewhat older than the diseased plants and consequently had slightly thicker stems. The difference in thickness was compensated for by adjusting the length of the stem section, so that a total volume of approximately 2.5 cc. was obtained. Table 14 shows the results of the measurements made with stems from 2 sets of diseased and recovered plants. The statistical analysis of the data is given in table 15. From these results no evidence was obtained that there is an essential difference in virus concentration of stems of recovered and diseased plants. The ratio between the mean square of the recovered *vs.* diseased stems and the experimental error is $34.4/13.3 = 2.6$, where the minimum value to be significant would have to be 3.9.

IX. Virus Concentration of Diseased Plants as Compared with Plants Grown through 10 Generations from Cuttings of Recovered Plants

In Section I of the paper, mention was made of the use of recovered plants grown through 10 generations from cuttings for the purpose of determining whether or not virus multiplies in recovered plants. Since these same plants were available for test, it seemed desirable to compare their virus content with that of diseased plants. Four tests were conducted in which the virus concentration of leaves of recovered plants grown through 10 generations from cuttings was compared with that of leaves showing acute symptoms of

ring spot. The results of the experiment are given in table 16 and an analysis of the data in table 17. The data show that recovered plants grown from cuttings had a considerably lower virus titer than diseased plants.

X. Virus Concentration of Recently Recovered Plants as Compared with Recovered Plants Grown through 10 Generations from Cuttings

Four tests were conducted in which measurements were made of the virus concentration of leaves from recently recovered plants and of leaves from recovered plants grown through 10 generations from cuttings. The data from these experiments are given in tables 18 and 19. They show that recovered plants in the 10th generation from cuttings contain considerably less virus than recently recovered plants. The ratio of the mean squares is 222.1 where 3.9 would be significant. The interaction is not significant.

The recovered plants obtained from cuttings appeared to grow less rapidly and were somewhat more stunted than the recently recovered plants. It is possible that the stunting may account for at least a part of the pronounced difference in virus concentration of the two groups of plants.

XI. Virus Concentration of Inoculated and Systemically Diseased Leaves

Three tests were conducted to determine whether or not inoculated leaves contain a higher virus concentration than systemically diseased leaves. In each test, measurements were made of the virus concentration in 10 inoculated and 10 systemically diseased leaves. The results of these experiments are presented in table 20. Table 21 gives the statistical analysis of the data.

The results show that the inoculated leaves in general contained more virus than systemically diseased leaves. The difference is clearly significant, the mean square $10{,}653.2 / 173.7 = 61.3$ where 6.7 would be highly significant. Examination of table 20 reveals that the inoculated leaves had consistently more virus than the systemically diseased leaves in both tests 1 and 3, whereas test 2 is anomalous in showing 8 out of 10 systemically diseased leaves with a larger virus concentration than their inoculated mates. In this connection it is well to consider in detail the methods employed in collecting the data. In test 1, each pair of inoculated and systemically diseased leaves was taken from the same infected tobacco plant. In test 2, the leaves making up a pair were taken from different plants and an effort was made to select systemically diseased leaves that showed as many necrotic lesions as shown by the inoculated member of the leaf pair. In the 3rd test, the leaves were from different plants and the samples of inoculum were prepared as follows: A large-size cork borer (number 6) was used to cut out a section of leaf tissue including a necrotic lesion. As a rule, only one lesion was removed with each disc of tissue, but in a number of instances more than one lesion was included. Ten such discs of tissue were cut from each of the leaves tested. This procedure was followed in an attempt to include the same number of necrotic

TABLE 13.—*Statistical anaylsis of the data summarized in table 12*

Sources of variation	Degrees of freedom	Sum of squares	Mean square
Between leaves of same plants	795	2,753.0	3.5
Between plants of same pot	595	6,935.9	11.7
Between pots of same inoculum	160	2,663.9	16.6
Between inoculum samples in same test and treatment	36	4,516.0	125.4
Variance based on pot means and adjusted for the harmonic mean number of lesions per pot			
Between tests	1	1,559.0	1,559.0
Between virus from recovered *vs.* diseased roots	1	645.9	645.9
Interaction	1	113.0	113.0

Harmonic mean number of leaves per pot is 7.34.

TABLE 14.—*Relative virus concentration of stems of recovered (R) and diseased (D) plants as indicated by the average numbers of lesions per leaf multiplied by 40*[a]

Plant No.	Test No. 1		Test no. 2	
	R	D	R	D
1	71	163	67	137
2	104	85	64	67
3	67	57	15	75
4	194	19	78	84
5	126	73	53	111
6	111	68	96	87
7	51	76	79	117
8	25	65	204	160
9	118	90	61	148
10	47	66	40	172
Total	914	762	757	1158

[a] See footnote, table 4.

lesions in each inoculum sample. The total quantity of leaf material used in test 3, therefore, was considerably less than that used in either test 1 or 2. This difference in quantity of leaf tissue accounts, in part, for the large variation between tests and also is reflected in the large value attributed to the interaction.

The mean difference in lesions per leaf between inoculated and systemically diseased leaves in test 1 is $12.44 \pm .7927$ (where $\pm .7927$ is equal to the standard error of this difference); in test 2 it is $-1.75 \pm .8829$; and in test 3 it is $1.70 \pm .3723$. The difference is highly significant in test 1, where the

TABLE 15.—*Statistical analysis of the data summarized in table 14*

Sources of variation	Degrees of freedom	Sum of squares	Mean square
Between leaves of same plants	810	2,812.0	3.5
Between plants in same pot	610	4,890.5	8.0
Between pots of same inoculum	160	2,121.9	13.3
Between inoculum samples in same test-and-treatment subclass	36	1,759.3	48.9
Variance based on pot means and adjusted for the harmonic mean number of leaves per pot			
Between tests	1	34.4	34.4
Between virus from recovered *vs.* diseased stems	1	34.4	34.4
Interaction	1	202.2	202.2

Harmonic mean number of leaves per pot is 8.83.

tobacco leaves were selected at random; it is not significant in test 2 and just barely significant in test 3, where selection was made on the basis of the numbers of necrotic lesions present in the tobacco leaves tested.

The data derived from the above experiment lead to the conclusion that inoculated leaves contain more virus on the average than do systemically diseased leaves and that this difference is, for the most part, accounted for by the difference in numbers of lesions to be found in inoculated and systemically diseased leaves. The effect of numbers of lesions on virus concentration is considered in more detail in Section XII.

XII. Virus Concentration of Leaves Inoculated with Different Dilutions of Ring-spot Virus

Two experiments were performed for the purpose of determining the effect of numbers of ring-spot lesions on virus concentration. The plan of the experiment was to determine the virus concentration of tobacco leaves inoculated with different dilutions of ring-spot virus. Five leaves of Turkish tobacco were inoculated with undiluted ring-spot virus, and 5 with virus diluted 1:5, 1:25, and 1:125. After 5 days an estimate was made of the numbers of lesions present in each of the 4 sets of leaves. The total numbers of lesions obtained were 1125 for the undiluted virus sample, 447 for the 1:5 dilution, 163 for the 1:25 dilution, and 36 for the 1:125 dilution. Measurements were then made of the virus concentration in each of the 4 sets of leaves. The experiment was repeated about a month later, but the numbers of lesions produced in the inoculated tobacco leaves were not recorded. Table 22 shows the numbers of lesions that were obtained from the 2 tests. An analysis of the data is given in table 23. The results show that the larger

the number of lesions present in a tobacco leaf, the greater, in general, is the virus concentration of that leaf.

The values for virus concentration are quite consistent in the 2 experiments, as indicated by the lower proportion of the variation contributed by the interaction. Considerable of the variation between leaves of diseased tobacco plants may, therefore, be attributed to the evident differences between the numbers of lesions present on such leaves.

DISCUSSION

By far the greatest contribution to the variation within the experiments reported in this paper is that between tests. This large difference results principally from the variation in susceptibility of the separate lots of cowpeas used as test plants and only partly from a variation in virus concentration of different lots of tobacco plants affected by ring-spot virus. That the latter do show considerable variation in virus concentration is demonstrated by the differences between inoculum samples within the same test-and-treatment subclass.

The evidence shows conclusively that recovery of tobacco plants from ring-spot disease is accompanied by a marked decrease in virus concentration. It is tentatively estimated that recovered leaves contain from 1/5 to 1/10 as much as actively-diseased leaves. The cells of recovered plants are not, as Valleau (10) has suggested, parasitized to the same limit as the cells of diseased plants.

TABLE 16.—*Relative virus concentration of leaves of recovered plants grown through 10 generations from cuttings (C) and recently diseased plants (D), as indicated by the numbers of lesions per leaf multiplied by 40*[a]

Plant No.	Test No. 1		Test No. 2		Test No. 3		Test No. 4	
	C	D	C	D	C	D	C	D
1	82	448	106	467	96	754	118	241
2	99	525	51	602	102	657	27	211
3	205	500	75	427	116	649	30	411
4	205	565	51	289	242	756	83	346
5	239	951	19	304	103	640	59	185
6	236	422	67	300	183	1023	196	355
7	9	195	72	158	140	511	92	386
8	302	570	35	378	252	885	211	387
9	24	359	76	203	127	762	58	447
10	45	272	230	152	202	573	178	289
Total	1446	4807	782	3280	1563	7210	1052	3258

[a] See footnote, table 4.

The difference in virus concentration between recovered and diseased plants affords a partial explanation for acquired immunity from tobacco ring

TABLE 17.—*Statistical analysis of the data summarized in table 16*

Sources of variation	Degrees of freedom	Sum of squares	Mean square
Between leaves of same plants	1,641	24,837.5	15.1
Between plants of same pot	1,241	93,565.1	75.4
Between pots of same inoculum	320	33,406.0	104.4
Between inoculum samples in same test and treatment	72	21,976.4	305.2
Variance based on pot means and adjusted for the harmonic mean number of leaves per pot			
Between tests	3	17,355.3	5,785.1
Between virus from diseased plants *vs.* plants grown from cuttings	1	55,277.6	55,277.6
Interaction	3	8,341.2	2,780.4

Harmonic mean number of leaves per pot is 7.76.

TABLE 18.—*Relative virus concentration of leaves of recovered plants grown through 10 generations from cuttings (C) and recently recovered plants (R), as indicated by the average numbers of lesions per leaf multiplied by 40*[a]

Plant No.	Test No. 1		Test No. 2		Test No. 3		Test No. 4	
	C	R	C	R	C	R	C	R
1	65	194	103	212	4	83	60	69
2	36	155	99	318	77	194	65	95
3	65	149	91	179	41	214	30	59
4	19	175	22	228	91	179	36	61
5	73	174	42	320	4	122	54	151
6	91	277	84	266	78	138	36	130
7	98	197	167	282	39	145	68	123
8	198	171	101	407	111	84	106	213
9	19	197	413	175	93	70	111	76
10	62	207	32	453	51	225	69	113
Total	726	1896	1154	2840	589	1454	635	1090

[a] See footnote, table 4.

spot. It does not, however, explain the mechanism for this phenomenon. That there must be a definite mechanism for regulating the multiplication of virus in recovered plants seems beyond question. Whether this mechanism involves the action of antibodies in the immune plants, or whether it is dependent on something else, is at present unknown. An hypothesis for re-

TABLE 19.—*Statistical analysis of the data summarized in table 18*

Sources of variation	Degrees of freedom	Sum of squares	Mean square
Between leaves of same plants	1,565	8,474.0	5.4
Between plants of same pot	1,165	17,064.0	14.6
Between pots of same inoculum	320	6,991.1	21.8
Between inoculum samples in same test and treatment	72	7,135.8	99.1
Variance based on pot means and adjusted for the harmonic mean number of lesions per pot.			
Between tests	3	3,359.7	1,119.9
Between recently recovered plants and plants grown from cuttings of recovered plants	1	4,841.9	4,841.9
Interaction	3	987.0	329.0

Harmonic mean number of leaves per pot is 7.23.

covery and acquired immunity from tobacco ring spot is as follows: The ring-spot virus is able to reach its maximum concentration in, and to exert its maximum effect upon, only those cells that are nearly mature at the time of invasion. The invasion of embryonic cells of tobacco plants is not accompanied by maximum increase in virus nor by severe injury to the cells. Having once been invaded, the embryonic cells become adapted to the presence of virus and maintain an equilibrium value with the virus. Symptoms are produced in tobacco plants only so long as the growing point has not been

TABLE 20.—*Relative virus concentration of inoculated (I) and systemically diseased (S) leaves as indicated by the average numbers of lesions per leaf multiplied by 40*[a]

Plant No.	Test No. 3		Test No. 2		Test No. 1	
	I	S	I	S	I	S
1	422	232	726	815	376	268
2	612	404	905	998	265	211
3	973	241	971	610	417	361
4	940	551	626	715	451	359
5	928	341	722	754	345	305
6	999	270	897	1307	384	283
7	756	524	895	1054	160	110
8	814	415	1009	1223	295	166
9	605	136	950	656	182	173
10	1471	374	651	878	165	88
Total	8520	3488	8352	9010	3040	2324

[a] See footnote, table 4.

TABLE 21.—*Statistical analysis of the data summarized in table 20*

Sources of variation	Degrees of freedom	Sum of squares	Mean square
Between leaves on same plants	1,253	31,290.5	25.0
Between plants in same pot	953	149,815.8	157.2
Between pots of same inoculum	240	41,683.0	173.7
Between inoculum samples in same test and treatment	54	46,238.2	856.3
Variance based on pot means and adjusted for the harmonic mean number of lesions per pot.			
Between tests	2	87,052.7	43,526.4
Between virus from inoculated leaves *vs.* systemically diseased leaves	1	10,653.2	10,653.2
Interaction	2	20,594.4	10,297.2

Harmonic mean number of leaves per pot is 7.91.

invaded. Once the embryonic cells become infected the plant recovers. The hypothesis is supported by the fact that the course of the disease may be prolonged or shortened by exposure of infected plants to good or poor growing conditions (5). Rapidly growing plants tend to grow away from the virus and required a longer time for recovery. When very young plants are inoculated, the virus rapidly reaches the tip of the plants and they quickly

TABLE 22.—*Relative virus concentration of leaves inoculated 5 days previously with certain dilutions of ring-spot virus, as indicated by the average numbers of lesions per leaf multiplied by 40*[a]

	Plant no.	1:125 Dilution	1:25 Dilution	1:5 Dilution	Undiluted
Test 1	1	107	353	563	425
	2	142	407	341	664
	3	124	893	937	821
	4	230	517	799	587
	5	146	188	583	723
	Total	749	2358	3223	3220
Test 2	1	31	202	295	145
	2	103	236	285	409
	3	96	89	278	307
	4	9	74	222	178
	5	25	120	326	585
	Total	264	721	1406	1624

[a] See footnote, table 4.

TABLE 23.—*Statistical analysis of the data summarized in table 22*

Sources of variation	Degrees of freedom	Sum of squares	Mean square
Between leaves on same plants	746	13,271.5	17.8
Between plants in same pot	546	55,370.5	101.4
Between pots of same inoculum	160	22,469.9	140.4
Between inoculum samples in same test and treatment	32	17,509.0	547.2
Variance based on pot means and adjusted for the harmonic mean number of lesions per pot.			
Between tests	1	14,266.3	14,266.3
Between virus concentrations	3	18,803.7	6,267.9
Interaction	3	2,038.2	679.4

Harmonic mean number of leaves per pot is 6.70.

recover from the disease. An objection to acceptance of the hypothesis is the fact that there is no definite proof that the embryonic cells at the growing point are ever invaded by virus.

The demonstration that virus multiplies in recovered plants raises the interesting question of where multiplication takes place. Ring spot is essentially a spot disease. It is well known that virus increase occurs in the primary lesions, and it has been assumed that increase also occurs in systemic lesions. The primary and systemic lesions are generally regarded as marking the areas of virus multiplication. No visible lesions occur in recovered plants, but virus has been found to multiply in these plants. There is at the present time little evidence regarding the locus of this multiplication. One possibility is that increase occurs in definite spots not marked by symptoms, and that virus moves from these spots into the growing point of the plant. Another possibility is that virus multiplies in the embryonic cells of the growing point of recovered plants, and that it moves into each new cell that is developed. The cells of the growing point may be capable of restricting the multiplication of virus to a level that is not sufficient to cause the production of lesions.

It is of interest that the decrease in virus concentration is confined to the leaves and roots, and that stems of recovered and diseased plants do not differ significantly in virus titer. The latter is not surprising, since stems never show symptoms of the disease. On the other hand, it is difficult to explain the difference between roots of recovered and diseased plants, since the roots have not been observed to show definite symptoms of ring spot. Perhaps the fact that, in the experiments reported, the roots of diseased plants were younger than those of recovered plants might account for the difference in virus concentration.

It is believed that recovery of tobacco plants from the ring-spot disease, accompanied by a marked decrease in virus concentration, and followed by immunity from reinfection, constitutes an acquired immunity comparable in many respects to that that obtains in virus diseases of animals.

SUMMARY

1. By growing recovered plants through a series of 10 generations by means of cuttings and subsequently testing for virus, it was shown that ring-spot virus multiplies in tobacco plants that have recovered from the ring-spot disease.

2. Using the number of necrotic primary lesions produced in leaves of Black cowpea (*Vigna sinensis* Endl.) as a measure of virus concentration, it was shown that leaves from diseased plants contain, on the average, from 5 to 10 times as much virus as leaves from recovered plants. The leaves of both recovered and diseased plants were found to vary considerably in virus concentration.

3. Measurements made of virus concentration in partly recovered leaves of tobacco plants affected by ring spot have shown that the healthy-appearing (basal) portions contain considerably less virus than the diseased (apical) portions of the same leaves. No evidence was obtained that apical and basal portions of either diseased or recovered leaves differ in virus concentration.

4. Fully recovered leaves were found to contain more virus than the healthy-appearing portions of partly recovered leaves.

5. The virus content of recovered leaves was not shown to have been increased by inoculating them heavily with ring-spot virus, although healthy leaves of the same age that were inoculated at the same time showed approximately 5 times as much virus as either the inoculated recovered or noninoculated recovered leaves tested 5 days after inoculation.

6. The roots of diseased plants were found to contain significantly more virus than the roots of recovered plants.

7. No evidence was obtained that there is an essential difference in virus concentration in stems of recovered and diseased plants.

8. Recovered plants grown through 10 generations from cuttings were found to contain much less virus than diseased plants. They were, likewise, found to contain somewhat less virus than recently recovered plants.

9. Leaves inoculated with undiluted virus contain, as a rule, more virus than systematically diseased leaves. This difference is partly accounted for by the numbers of necrotic lesions present in such leaves.

10. Measurements made 5 days after inoculation of tobacco leaves with various dilutions of ring-spot virus showed that the larger the number of lesions present in an inoculated leaf the greater, in general, is the virus content of that leaf.

11. Cowpea plants used for testing virus concentration were found to vary considerably in susceptibility to ring-spot virus, the variation being greater in plants grown under different conditions than in those grown under the same conditions. Variation between leaves on the same plants was found to be less than that between leaves on different plants.

FROM THE DEPARTMENT OF ANIMAL AND PLANT
PATHOLOGY OF THE ROCKEFELLER INSTITUTE FOR
MEDICAL RESEARCH, PRINCETON, NEW JERSEY

LITERATURE CITED

1. FISHER, R. A. Statistical methods for research workers. 4th ed. 307 pp. Oliver and Boyd, Edinburgh. 1932.
2. FROMME, F. D., and S. A. WINGARD. Blackfire or angular-leafspot of tobacco. Virginia Agr. Expt. Sta. Tech. Bull. 25. 1922.
3. FROMME, F. D., S. A. WINGARD, and C. N. PRIODE. Ringspot of tobacco; an infectious disease of unknown cause. Phytopath. **17**: 321–328. 1927.
4. KÖHLER, E. Untersuchungen über die Viruskrankheiten der Kartoffel. III. Weitere Versuche mit Viren aus der Mosaikgruppe. Phytopath. Ztschr. **7**: 1–30. 1934.
5. PRICE, W. C. Acquired immunity to ring-spot in Nicotiana. Contrib. Boyce Thompson Inst. **4**: 359–403. 1932.
6. SAMUEL, G., and J. B. BALD. On the use of the primary lesions in quantitative work with two plant viruses. Ann. Appl. Biol. **20**: 70–99. 1933.
7. SMITH, K. M. The present status of plant virus research. Biol. Rev. and Biol. Proc. Cambridge Phil. Soc. **8**: 136–179. 1933.
8. SNEDECOR, G. W. Calculation and interpretation of analysis of variance and covariance. 96 pp. Collegiate Press, Inc., Ames, Iowa. 1934.
9. VALLEAU, W. D. Seed transmission and sterility studies of two strains of tobacco ringspot. Kentucky Agr. Expt. Sta. Bull. 327: 43–80. 1932.
10. ————. Do tobacco plants recover and develop an immunity from ring spot? (Abs.) Phytopath. **25**: 1935.
11. WINGARD, S. A. Hosts and symptoms of ring spot, a virus disease of plants. Jour. Agr. Res. [U. S.] **37**: 127–153. 1928.
12. YOUDEN, W. J., and H. P. BEALE. A statistical study of the local lesion method for estimating tobacco mosaic virus. Contrib. Boyce Thompson Inst. **6**: 437–454. 1934.

BLACK ROT OF TOMATO, LYCOPERSICUM ESCULENTUM, CAUSED BY ALTERNARIA SP.[1]

ELSBETH E. WARNER[2]

(Accepted for publication July 8, 1935)

INTRODUCTION

In the black rot of tomato the spots either appear on the outside and progress toward the center or they appear in the center and progress toward the outside, frequently without showing any signs of unsoundness on the surface. Investigation revealed that the rot was caused by a fungus, *Alternaria* sp. Although various species of Alternaria have been studied by others, this investigation has brought to light some unexpected characteristics in the life history of the species of Alternaria causing this disease, and some results have been obtained that may indicate the method of infection.

HISTORICAL REVIEW

Douglas (1) found that the alternaria disease of tomato appeared after the first fall rains and generally increased until the end of the season, and secured infection by placing drops of spore suspensions "on well-sized, green fruits just previous to the time they began to change color." Characteristic alternaria spots also were produced on the leaves of some varieties of tomato by placing them in a moist chamber and spraying them with a suspension of the spores in sterile water. The leaves of certain varieties did not yield to this method of infection.

Gibson (2) reported *Alternaria atrans*, severely infecting soy beans and cow peas, following injury by sunburn and aphid bites.

Guba (3) found that the viability of spores of *Alternaria dianthi* was destroyed after 2 years in the open field.

Martin (4) reports that *Alternaria solani* spores were carried, not only on the bodies but also in the excreta of 4 different species of insects that customarily visit the tomato. Many spores were found also on the hands and garments of pickers.

Pool (5, p. 3–6) studied a disease of tomato caused by *Alternaria fasciculata*, which displayed symptoms similar to those of the disease here discussed, although the fungus which she describes differs widely from that of the present discussion.

Whetzel (6) found severe infection on potatoes caused by *Alternaria* sp. following a rainy season.

[1] This work was done in partial fulfilment of the requirements for the Master of Arts degree in Plant Pathology, in Oberlin College.

[2] The writer acknowledges her indebtedness to Dr. S. P. Nichols and Prof. George T. Jones for their valuable advice and criticism during the course of the investigation.

MATERIALS AND CONDITIONS

The present investigation was begun in the open field during the growing season of 1934, in connection with other experiments on tomato. The summer was very hot and dry, making frequent cultivation necessary for the conservation of the soil moisture. None of the plants was watered artificially. They were completely surrounded by corn except for a distance of about 2 rods where peas, peppers, and bush beans were planted in adjacent space. The competition of the corn for the scant water supply doubtless contributed to the dryness of the soil.

The tomato vines were carefully staked to protect the fruit from contact with the soil. In this connection it should be noted that the tomatoes in surrounding or near-by gardens were not staked, the fruit being allowed to lie on the ground. In these gardens the infection was much more severe than in the plot where the experiment was being conducted and where the disease was rather infrequent.

The prevailing winds were S.W. to N.W. and N.E., and it is interesting here to note that the tomatoes on the north end of the garden were most severely infected. Attention should be called to the fact that at this end of the garden the corn was so stunted as to be hardly taller than the tomato plants themselves.

Varieties used in the field were Marglobe, Ponderosa, and Pritchard. Heaviest infection appeared on the Marglobe, but its greater susceptibility to the disease has not been definitely established. In previous years other varieties planted in the same area always showed some infection.

SYMPTOMS

Infection occurs on both green and ripe fruit, and is confined to the fruit. The infection spots are not restricted to one location on the fruit, but are more generally found externally at the blossom end (Figs. 1 and 2, A). The infected areas are slightly wrinkled, dark brown, and, in their later stages, develop velvety mats of conidiospores, as observed by Douglas (1). The spots range in size from minute pinheads to areas extending completely across the surface of the fruit, giving it a flattened face. After the diseased fruit had been separated from the vine and exposed to dampness a grayish white mycelial growth appeared on the lesions rapidly spreading over the entire fruit (Fig. 2). If the infected areas are internal with no evidence of the disease on the surface of the fruit, the spots range from 1 to 2 inches in diameter and are pitch black. This internal rot is dry and leathery. The underlying tissue of the more severely infected portions of the spots is extremely tough, and is, therefore, very difficult to section. Free-hand sections, made with a safety-razor blade, showed that the most recently infected

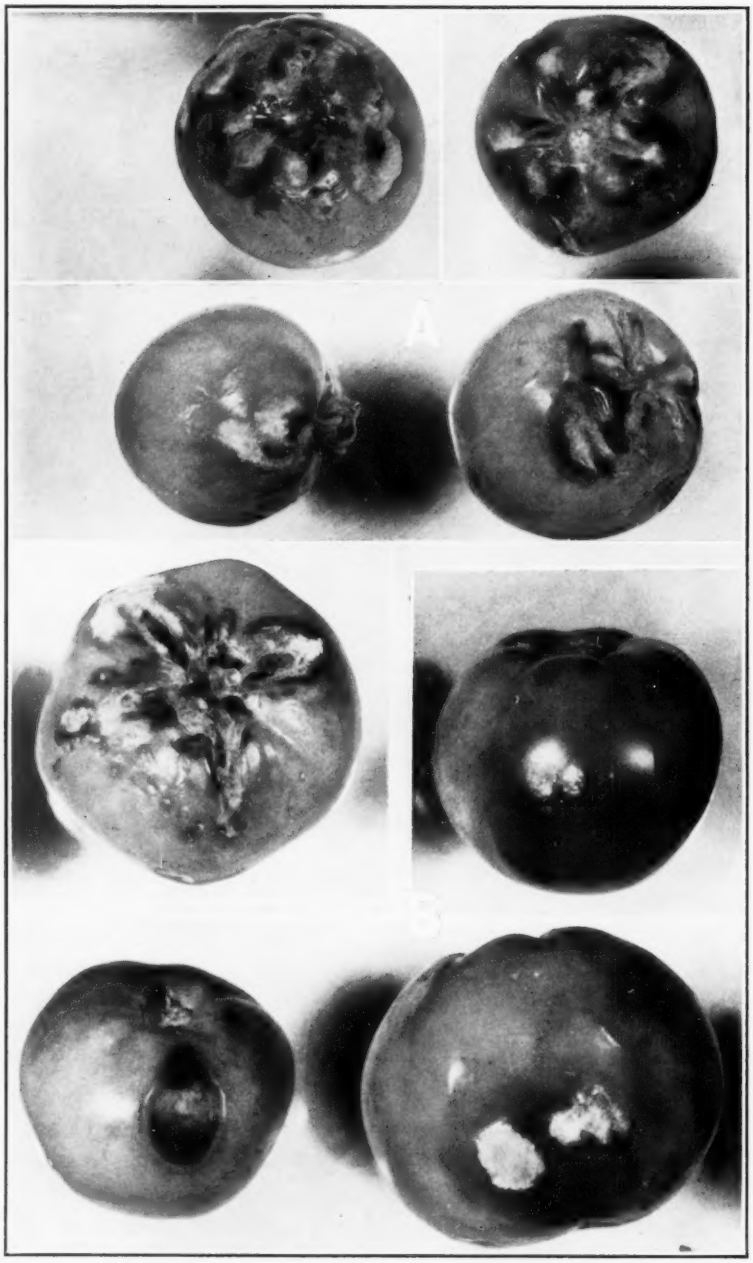


FIG. 1. A. Stem-end infections, resulting from inoculations through mechanical wounds, while the fruit was still on the vine. B. Lateral infection resulting from stab while fruits were still on the vine.

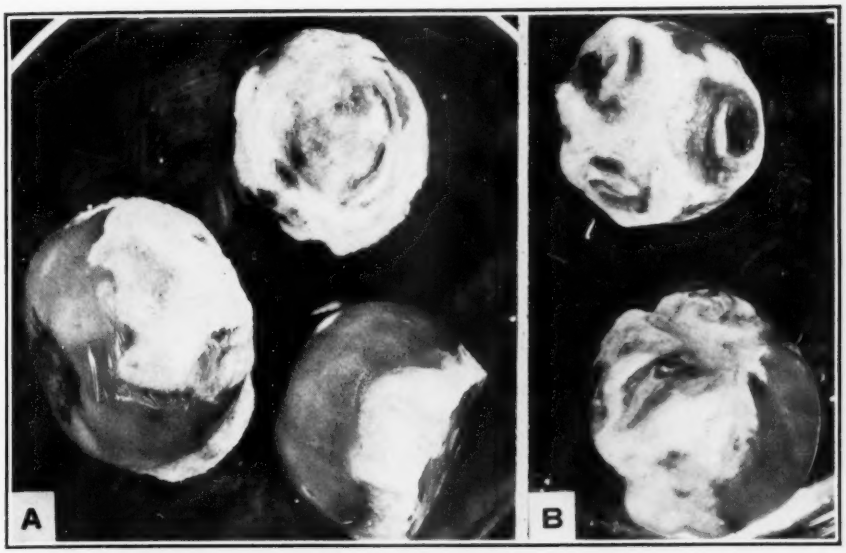


FIG. 2. A. Blossom-end infection. Infection developed on the vine and fruits were later placed in moist chamber, bringing out the gray mycelial growth over the infected areas. B. Mechanical injuries produced on fruit off the vine. Fruits kept in moist chamber, resulting in mycelial growth.

tissues were tan to brown,[3] while the region of severest infection was dark brown to black. Microscopic examination revealed a dense mass of dark brown hyphae in the older areas, with brownish to green to almost hyaline filaments penetrating the surrounding healthy tissues. These symptoms differ from those published by Pool (5) who found the hyphae to range from black in the older areas to brownish in the areas of least infection.

In order to make microtome sections of the tomatoes, slices were made through regions of infection and of noninfection. These were killed by submerging them in chromo-acetic acid for twelve hours. The slices were then washed under running water for twelve hours. After washing they were run through the standard method prescribed for imbedding tissues in paraffin.

Microtome sections were made of diseased tissue and stained with Fleming's triple stain. This proved to be unsatisfactory, however, so the sections were stained 5 minutes in safranin and 15 seconds in orange G with decidedly better results.

A strange fact is that Delafield's haematoxylin, so highly recommended for this type of tissue, proved quite unsatisfactory, even when sections were left in the stain for 4 days.

[3] The colors mentioned in this discussion are named in accordance with "Color Standards and Nomenclature" 1912, by R. Ridgeway.

The use of Fleming's triple stain on microtome sections disclosed the fact that the hard portions of the tissue were made up of very compactly arranged host cells gorged with hyphae. The cell walls were frequently badly

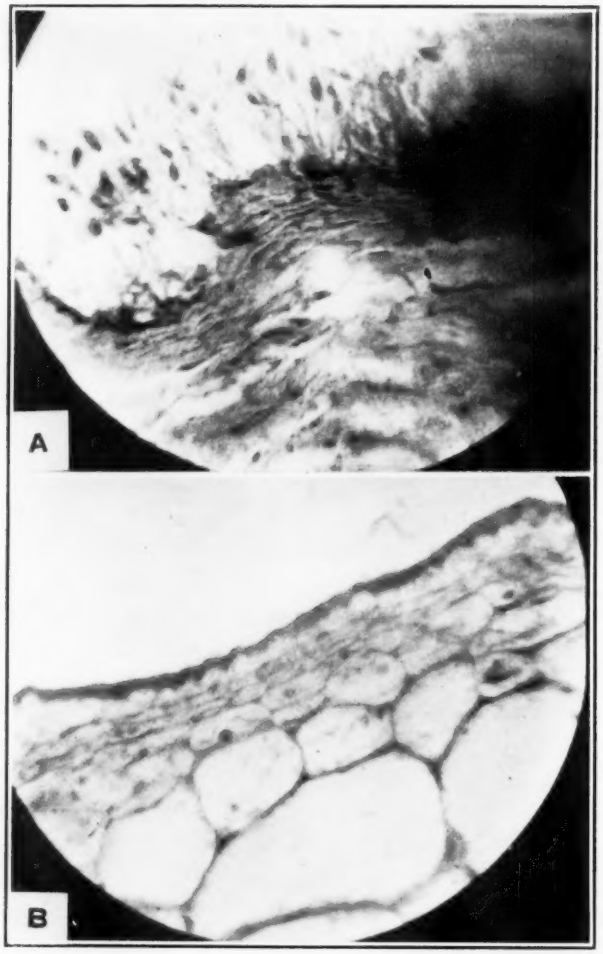


FIG. 3. Sections of tomato tissue. A. Diseased. B. Noninfected.

broken, and, where not broken, they had thickened considerably. The fungus frequently digests and absorbs the host tissue, walls as well as protoplasm, leaving large open spaces, thus causing the infected area to collapse and wrinkle in spots (Figs. 3 and 4). The transition from the infected area to the noninfected is a gradual one. In this respect the results differ from those of Pool (5), who found the transition to be very abrupt.

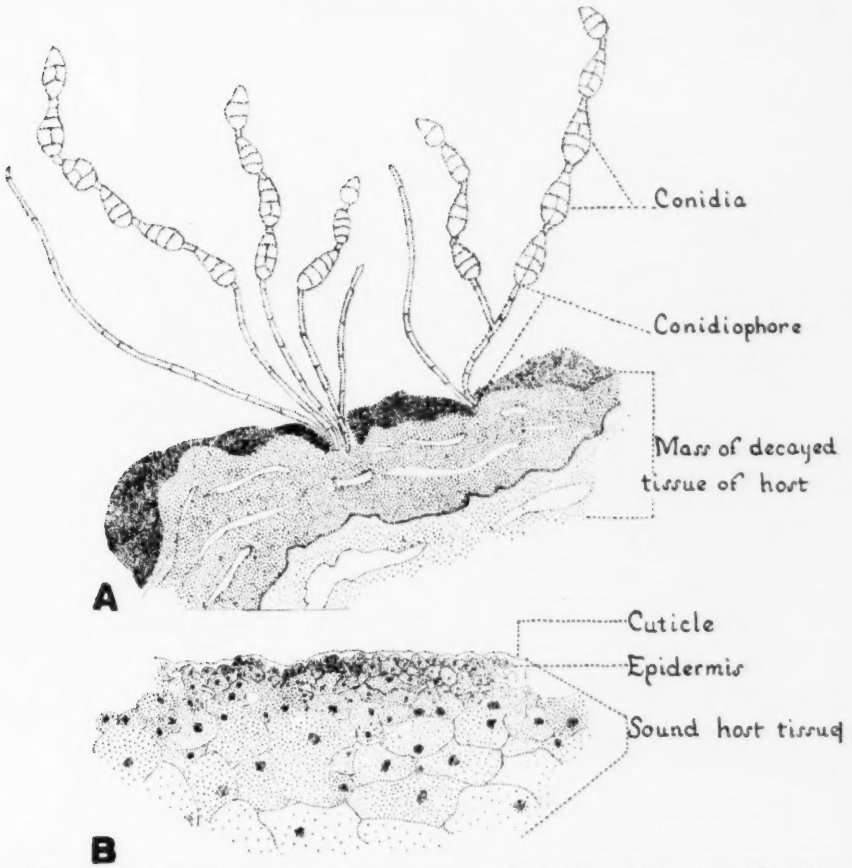


FIG. 4. Details from figure 3. A. Clusters of sterile and fertile conidiophores arising from disorganized and diseased host tissue. B. Noninfected host tissue. Both $\times 640$.

THE FUNGUS

Morphology

For the study of the fungus it was necessary to isolate it and grow it in pure culture. Potato agar, potato-dextrose agar, nutrient agar and tomato agar were used. Standard recipes were followed for all of the agars except the tomato. Growth on all but the tomato agar was unsatisfactory. On both potato and potato-dextrose agars the growth was black and formed a thin mat. On nutrient agar it was extremely slow, the mat being only 2 inches in diameter after 3 weeks. Growth on the tomato agar was a decided contrast, producing in from 3 to 5 days an average mat of 2 inches in diameter. After much experimentation it was found that the best agar was obtained when the following recipe was used: 30 grams of agar to 1000 cc. of

strained tomato juice. Although 30 grams seems an unusually large amount of agar for only 1000 cc. of juice, it was found that a less amount would not gel the liquid. The agars were all sterilized at 15 lb. pressure for 20 minutes.

Plate cultures made from portions of the diseased tissue resulted in pure cultures of the Alternaria. Inoculations made from these plate cultures on

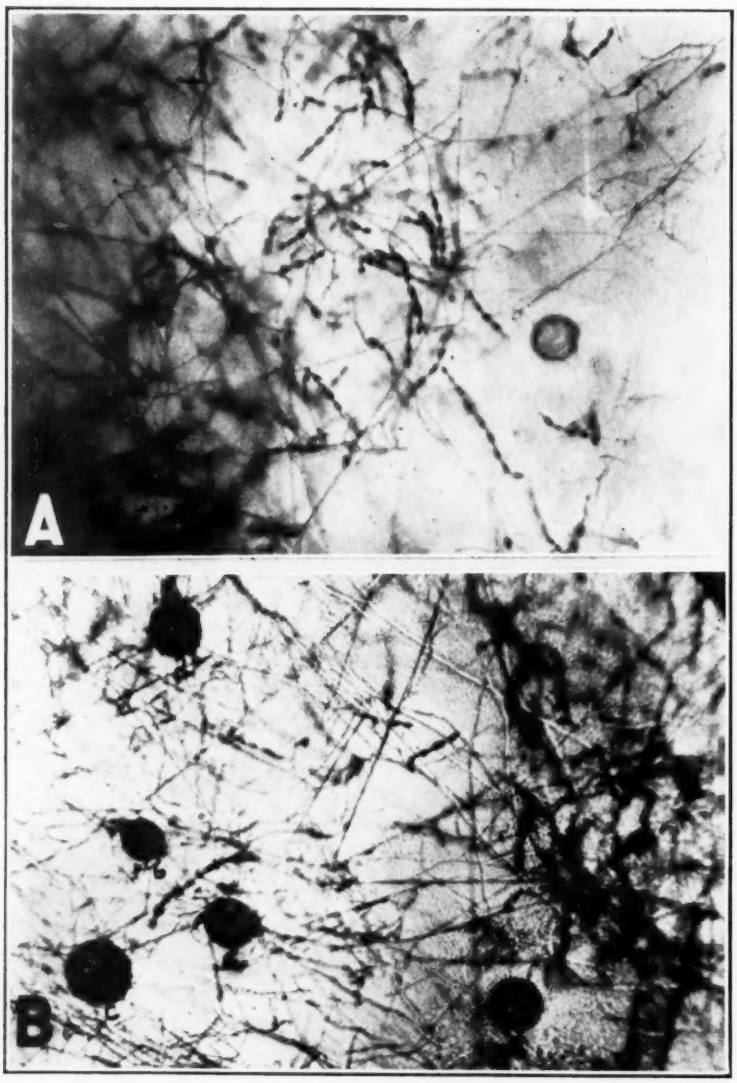


FIG. 5. A. Types of conidial chains. B. Large resting spores (a, b, c, d, e).

noninfected tomatoes reproduced the disease in every respect. Plate cultures made from these artificially infected fruits showed the organism to be the same as that previously isolated.

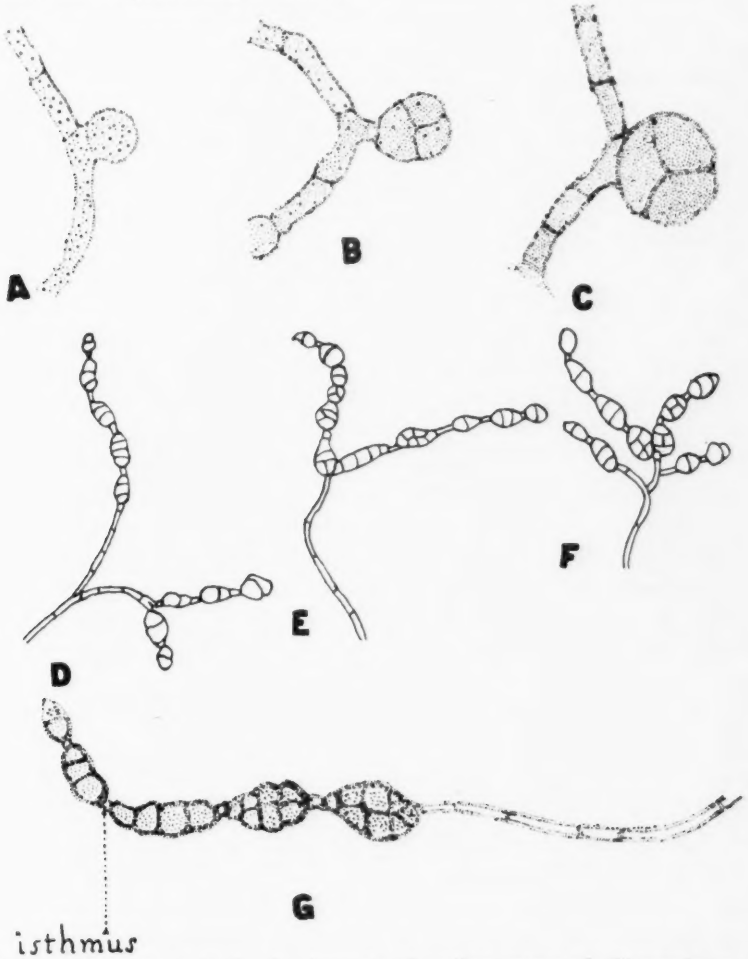


FIG. 6. A–C. Stages in the development of resting spores of *Alternaria* sp. D–G. Details of conidial branching from figure 5, A. A–C, G. ×500. Others drawn under low power.

Microscopic examination of the fungus revealed that the fertile hyphae are at first hyaline to green, becoming tan to dark brown as the conidia form. Sterile filaments are always of a lighter color, frequently remaining hyaline throughout their life. Conidiophores, when mature, remain tea green and measure 59.2 to 74.0 μ by 1.6 to 2.2 μ. Conidia are dark brown, measuring 20.8 to 36.0 μ by 3.2 to 9.6 μ. These measurements differ from those of

Pool (5), which were much larger. Her conidia measured 35 to 66 μ by 16 to 20 μ, while her conidiophores measured 30 to 40 μ by 4 to 5 μ, and both her conidiophores and conidia were dark brown. The conidia in the experiment under consideration had 3 to 6 transverse and 1 to 3 longitudinal septa. The apical cell or isthmus connecting conidium to conidium was hyaline to dark brown, measuring 0.2 to 0.5 μ in length (Fig. 5, A), often producing a side branch that formed from 1 to several conidia (Fig. 5, A and D–F). These side branches occurred frequently, being very characteristic. The conidial chains comprised conidia numbering 1 to 11 on a single branch. Pool (5) speaks of both her conidia and hyphae as being prominently echinulate, but the species here considered showed no tendency toward echinulation.

When the organism in the petri-dish cultures had almost completely digested the medium, large, round, four-cell spores 9.6 to 12.3 μ in diameter appeared. These were dark brown with very thick walls (Fig. 5, B, and Fig. 6, D to G). These spores never appeared unless the food became scarce. Time did not permit testing the fungus to determine whether the spores would develop as a result of merely lowering the temperature. But if these spores, which in later discussion are described as resting spores, were not present when a culture was placed in a freezing refrigerator, no matter how abundant the conidia, transfers from them showed no growth.

The author has not identified this species because none of the obtainable discussions of species already identified corresponded to the characteristics of the species under discussion. The Alternaria with which we are here concerned differed from published accounts of species in the following characteristics: (1) The number of conidia in a chain, which range from 5 to 11 as compared with 3 to 5 in species studied by others; (2) the isthmus is much shorter in comparison with that of other species; (3) the conidia are much smaller on an average and are less septate than those of many previously described species; (4) there is an abundant production of both conidia and resting spores on artificial media as well as on host tissue; (5) the fact that this species has two types of spores, while other species of Alternaria have only one. Whether this fungus is a new species or whether its apparent variations are merely reactions to environmental conditions, is a question that must be left to later investigation. It has been frequently observed that fungi, grown on different media or on different hosts, show considerable variation. The differences have been so pronounced that they have frequently been mistaken for distinguishing characteristics of new species. Therefore, before calling this Alternaria a new species rather than a habitat form, it would be necessary to grow it in varying environments and on different hosts.

However, one of the characteristics disclosed by this experiment is im-

portant enough to call for further discussion. Alternaria is not known to have differing types of spores, namely, conidia and resting spores. When this condition was first noticed it was thought that the culture was impure. To test this the following method of making plate cultures was followed. Shallow Petri dishes were poured with a layer of agar as thin as was possible and still cover the entire surface; with such a thin layer of agar the plate was quite transparent. Since dissecting needles become much roughened on the surface as the result of frequent flaming a new needle was used. It was sterilized in a 1-to-1000 solution of mercuric chloride. With this needle young cultures of Alternaria were barely touched and the needle thus infected was then used to streak the agar in the shallow plates above described. Examination of these plates by the low power microscope showed from 3 to 4 conidia in a streak, without any hyphae or other fungus material.

These plates were carefully checked from day to day and the spores were seen to germinate and grow. After extensive growth of the mycelium and abundant production of ordinary conidia, all of the 25 plates infected in this manner gave rise to round spores as soon as the agar was almost entirely digested. These plates were then frozen for 3 months, which is time enough to kill the conidia. Transfers made from the cultures produced the characteristic Alternaria conidia and again production of these resting spores when the food became scarce (Fig. 5, B).

PHYSIOLOGY

A number of agar-plate cultures were constantly maintained for experimentation. Tube cultures proved unsatisfactory for this fungus, because of the small surface of exposed medium. In order to observe closely the growth of the fungus from day to day, shallow Petri dishes were covered with a thin film of tomato agar and inoculated by either the streak or stab method. These Petri dishes were then placed in moist chambers and kept in a room-temperature incubator. Out of 800 plate cultures, pure cultures were obtained on all but 10 plates. These 10 were contaminated by *Rhizopus nigricans* or a Penicillium, introduced, no doubt, from another part of the laboratory where work was being done on these particular fungi.

To determine reaction to various environments, cultures were grown in light and dark places in the room where the temperature fluctuated between 15° and 24° C. Others were placed in the blood-heat incubator, room-temperature incubator, and in the refrigerator at 10° to 15° C.

Plates placed in the blood-heat incubator showed little or no growth. The mats increased only about 3.5 mm. in diameter before the culture died.

Cultures in the room-temperature incubator and those kept in the dark in the room where the temperature fluctuated from 15° to 24° C. grew satisfactorily. The type of growth of the cultures kept in the dark in the room

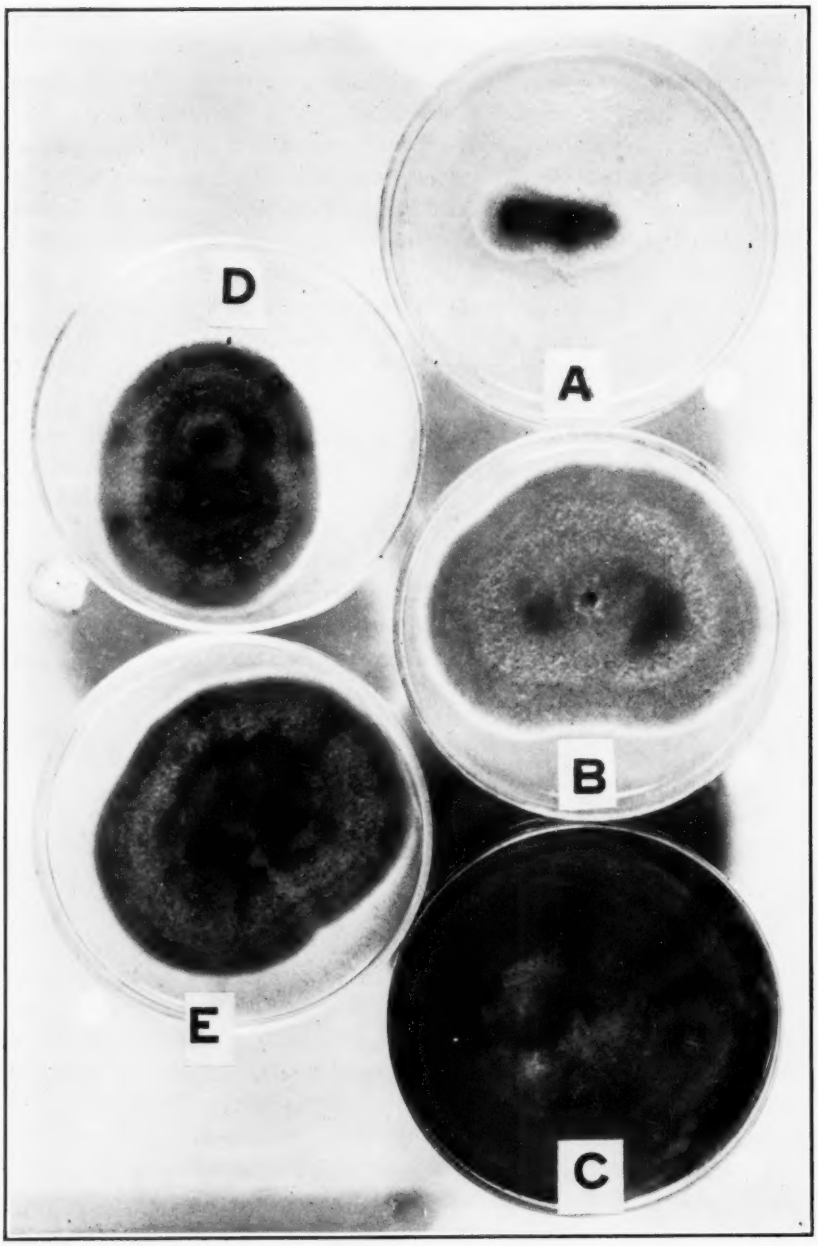


FIG. 7. Cultures of Alternaria. A. 6 days in incubator at room temperature. B. 2 weeks in incubator at room temperature. C. 6 weeks in room temperature (15°–24° C.) D. 10 days growth in room temperature (15°–24° C.) E. 2 weeks growth in room temperature (15°–24° C.).

was the same as that of those kept in the room-temperature incubator. The only difference was that the cultures grown in the room with fluctuating temperature showed a decided tendency to a variegated type of coloring. As long as the temperature remained low, growth was slight and a deep green. On the other hand, while the temperature was high, there was a pronounced increase in growth and a decided change in color from deep green to light tea green. The change from one color to another would be abrupt or very gradual, depending on the rapidity with which the temperature changed (Fig. 7, B–E).

The development of cultures placed in the light in the room was very similar to that of those grown in the dark, the only difference being in the size of the mat. Those grown in the light were about 2 cm. smaller in diameter, but the variegated coloring was still evident.

In the Petri dishes kept under the fluctuating room temperature the fungal mat of the Alternaria when 6 to 20 days old had a white margin, merging into tea green to dusky yellowish green towards the center. The very center was dusky olive green to black with gray to whitish wefts, either scattered over the entire surface or forming a complete border around it. The author was unable to determine the reason for this variegated appearance of the fungal mat when grown under conditions of variable room temperature. There was no apparent effect upon the hyphae except the change from deep green to tea green, according as the temperature was low or high.

Cultures kept in the refrigerator at 10° to 15° C. made little or no growth, and apparently none at all at 10° C. Some slight growth occurred at 15°; but in only 15 or 20 cultures did the mycelial mat increase. The growth was slow and unnatural in appearance, being pale green; and at the end of the 3 weeks during which they were kept in the refrigerator the average diameter of the culture was about 2 inches, or about 1/9 of what the growth would have been at room temperature.

An experiment was planned to determine the conditions in which the fungus overwinters. Cultures that had been grown in the room-temperature incubator for various lengths of time, ranging from 6 days to 6 weeks, were placed in the refrigerator and kept at −5° C. for periods of 7 to 90 days (Fig. 7). The results obtained are shown in table 1.

In experiments 4 and 5 (Table 1), in which growth was obtained on 27 of the 150 transfers, it is to be noted that these came from 9 of the 50 original plate cultures. These 9 cultures were grown in very shallow Petri dishes that held about 1/3 as much medium as other plates in the same experiment. In experiment 6 in which 6 out of 75 transfers failed to show growth, it should be noted that all 6 of these transfers were taken from 2 extra-deep original plates, holding about twice as much medium as those used in the remainder of the experiment. Consequently, all the agar was not entirely consumed.

TABLE 1.—*Results of transfers made from cultures of Alternaria sp. placed in the refrigerator*

Experiment No.	No. of plates used	Days under growth conditions at start of exper.	Days in refrigerator	No. of plates from which transfers were made	Total transfers made	No. of transfers showing growth	Percentage of transfers showing growth
1	25	6	7	25	75	0	0
		10	7	3	9	0	0
2	25	10	24	3	9	0	0
		10	90	19	57	0	0
		14	7	8	24	0	0
3	25	14	24	8	24	0	0
		14	90	9	27	0	0
		21	7	8	24	0	0
4	25	21	24	8	24	0	0
		21	90	9	27	3	11.1
		28	7	8	24	0	0
5	25	28	24	8	24	12	50
		28	90	9	27	12	44.4
		42	7	8	24	24	100
6	25	42	24	8	24	21	85.5
		42	90	9	27	24	88.8

Guba (3) reported that the viability of *Alternaria dianthi* extended over a period of only 2 winters. The evidence presented in table 1 shows that neither the mycelium nor the conidia of the species under discussion could survive even a week of freezing. This would indicate the necessity of a winter-hardy spore, capable of germinating on the restoration of favorable growing conditions. Such, apparently, is the function of the large, round resting spores, described in the foregoing. The absence of these spores explains the lack of growth in the 225 transfers made in experiments 1, 2 and 3, in that the young cultures from which the transfers were made, had produced no resting spores and the conidia were all killed by freezing. It explains also the large percentage of growth in the transfers made from the plates in experiments 5 and 6 in which enough time had been given for the exhaustion of the food supply and the consequent formation of resting spores. These spores were visible before the cultures were placed in the refrigerator. If the resting spores develop as the food becomes scarce, it would appear that, under natural conditions, they must be formed as the tomato fruits become more completely broken down and the available nutritive materials become exhausted by the fungus.

Plate cultures made from fruits frozen in the refrigerator showed no growth when the tomato used as the source of infection was only slightly diseased. However, when the plates were inoculated from thoroughly diseased fruits, 15 out of 25 showed growth. The explanation of the failure to produce growth when the disease is only slight lies in the fact that food is still abundant, no resting spores have been formed and none of the fungus is in condition to endure freezing. On the other hand, when the decay is considerable and the food supply in at least part of the fruit has given out, the resting spores are formed and freezing is not fatal to the fungus. Dissection of tissue from these thoroughly diseased fruits to determine the presence of resting spores was difficult because the tissue was extremely tough and not easily separable into thin sections. It was possible, however, to locate enough of the spores to verify their presence.

INOCULATION EXPERIMENTS

During the summer disease-free ripe fruits were removed from the vines and inoculated from Petri dish cultures by mechanical injury and insertion of mycelium. 5 to 6 days after inoculation these ripe fruits showed rotted areas measuring from $\frac{1}{2}$ to $\frac{3}{4}$ in. in diameter. In from 10 to 12 days the same fruits were rotted through $\frac{1}{3}$ to $\frac{3}{4}$ of their entire extent. Infected tissue thus produced resembled that produced by natural means in every respect, being hard, leathery, collapsed, and blackened.

Green fruits, also picked from the vines and inoculated in the same manner, required from 5 to 9 days longer than the ripe ones for the same amount of infection and decay to result, but they manifested the same characteristics, being hard, leathery, collapsed, and blackened. The only difference between the appearance of the disease on the green fruit and that on the ripe was that the ripe fruit was more wrinkled on the surface and the tissue was more collapsed.

Fruits inoculated in the same way, while still on the vine, responded in much the same way as did those inoculated after picking, the only difference being in the time necessary to bring about the same severity of infection. In the case of ripe fruit on the vine it required 8 to 10 days longer to develop the same amount of decay as obtained in ripe fruit inoculated after removal from the vine. In the case of green fruit on the vine, an infection equaling that of green fruit off the vine took from 10 to 15 days longer to develop.

Evidently, the decay is more rapid in ripe fruit than in green and more rapid in picked fruit than in non-picked (Fig. 8). Removal of the fruit from the vine lowers its resistance and makes possible a rapid penetration of the mycelium, probably because the characteristic cell turgidity is disturbed and the protoplasmic resistance is gradually overcome. The same would hold for ripe fruit as compared with green, except that, in the case

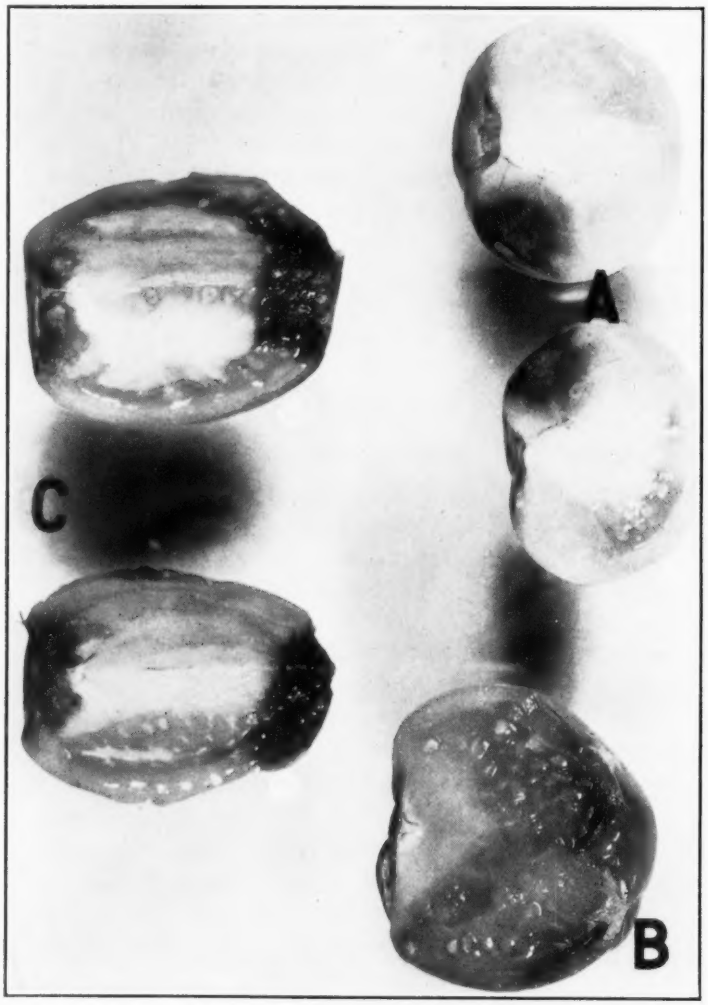


FIG. 8. Longitudinal sections through infected fruit inoculated after picking. A. Green fruit 12 days after inoculation. B. Ripe fruit 12 days after inoculation. C. Ripe fruit 5 days after inoculation.

of the ripe fruit, the cell walls have become more permeable, thus facilitating decay.

During the fall and winter, greenhouse experiments were performed to determine the method of infection under natural conditions. Seed was selected in the following manner. Two fruits were chosen, one of which was perfectly sound and the other decayed. They were placed in individual dishes to avoid contamination of the seed of the sound fruit by contact with

the infected fruit. The seeds of each were washed from the pulp. Since abortive seeds will float with the bulk of the pulp, sound seeds were easily obtained from among those that sank. A much larger proportion of the seed from the decayed fruit floated and was discarded. Seeds from the sound fruit, after being treated with a 1/10 per cent solution of mercuric chloride to reduce loss from various fungi, were planted in rich soil. The seed from the decayed tomato was not disinfected with mercuric chloride, since the spores of the infecting Alternaria might be carried on the seed.

The best 54 seeds from each source were selected for planting. One hundred per cent germination and growth occurred with the seed from the sound tomato, while only 22 per cent of that from the decayed tomato produced plants.

Microscopic examination of sectioned seeds showed that in some of the seeds the fungus had completely digested all the endosperm and almost all the ovule. In other seeds the infection was not so severe, for only a part of the endosperm and none of the ovule was absorbed. Probably the infected seeds that did produce plants were diseased only partially or not at all, while in those that germinated but were too weak to produce plants, the fungus had absorbed so much of the endosperm that there was not sufficient food left to support the seedling.

Eleven plants from seed of infected fruit and 11 from seed of non-infected fruit were planted in individual 8-in. pots of sandy loam. Soil in 6 pots of each of these 2 groups was inoculated by pouring on to it sterilized water to which had been added conidia of Alternaria. The remaining 5 plants in each group were reserved as controls.

One cluster of fruit on each of the 22 plants was bagged with cellophane during the entire period from the appearance of the blossom clusters until the ripening of the fruit. Another cluster on each plant was bagged only during blossoming; the bags were removed as soon as the fruit had set. When these bagged fruits were ripe they were carefully sectioned and in no case was the infection found. Evidently, neither infected seed nor infected soil is directly responsible for the black rot of the tomato.

In order to secure more normal growth the tomato plants were artificially lighted 14 hours daily. This not only resulted in stronger, stockier plants, but helped to shorten the time between seed planting and fruit production. The plants were carefully pruned to prevent excessive branching and were staked to facilitate more uniform exposure to light. Some branches were allowed to grow, but not enough to shut off light from the lower leaves.

Because of limited time the experiment in the greenhouse was made only with the variety Marglobe, chosen because of its short stigma, which would greatly facilitate self-pollination and therefore the setting of fruit in the absence of pollinating insects, and also because it had been most heavily infected in the open field during the preceding summer.

Fruits not bagged were contaminated by some one of the following procedures: painted stigmas, stab, surface contact or dust infection. In painting the stigmas sterile horsehair brushes were brushed over pure cultures of the Alternaria fungus. The brushes thus contaminated were then rubbed repeatedly over the ends of stigmas that were not over 3 days old. Most infection was received when the stigmas thus painted had been exposed to the air for only a few hours or for a day. Little or no infection occurred after the stigma was 3 days old.

Stab infection was accomplished by means of sterile needles, which, after being contaminated by scratching the pure culture of Alternaria, were used to prick or scratch the green or ripe fruit while still on the vine.

In preparation for dust infection an average-size frozen fruit, decayed by the fungus, was ground up with 300 grams of sterile soil. This mixture, after being thoroughly air-dried, was blown on newly opened flowers. The rest of the plant and other plants were carefully shielded from dust by means of newspaper caps.

Surface contact was secured by placing some of the fungus on the surface of the fruit without injuring the epidermis. Since the purpose of this second phase of the experiment was to duplicate in the greenhouse the conditions of the preceding excessively dry summer, to determine how the disease was spread, water suspensions of spores were not placed on the surface of leaves or fruit. This was because of the fact that in the garden during the preceding summer the moisture was so slight that for several successive weeks there was no dew. Douglas (1) found that infection occurred if the spores were suspended in water when placed on the surface of the fruit. He did not try the dry method of contact.

Leaves and stems of the tomato, inoculated by the same procedure as that followed in the inoculation of the fruit, gave negative results. Leaves and stems apparently are immune from the disease. Douglas (1) found that in some varieties these organs were susceptive to alternaria infection, while those of others were not. Marglobe may be resistant.

Results of the experiment with the growing fruit are shown in table 2.

Since none of the control clusters of fruit, whether from infected seed or not showed any spontaneous development of the disease whatever, it would appear that infection is not spread by infected seed. The fact also that these control clusters were not infected, even though many other clusters on each plant were heavily so, would indicate that the disease cannot pass from cluster to cluster through the stems. This opinion is strengthened by the fact that it was impossible to grow the fungus on the stems or leaves, regardless of the method of inoculation.

From table 2 it would appear that mechanical injury is most effective, since this method was 100 per cent efficient. But the possibility of any

TABLE 2.—*Results obtained from artificially infected fruits*

Method of treatment	No of fruits treated	No. of fruits infected	Percentage of fruits infected	No. of fruits not infected	Percentage of fruits not infected
Stab	55	55	100	0	0
Painting stigma	49	39	79.4	10	20.6
Blown dust	42	25	59.5	17	40.5
Surface contact	20	0	0	20	100
Control bagged	25	0	0	25	100
Control not bagged after formation of fruit	45	0	0	45	100

great amount of mechanical injury in the field, under natural conditions, especially when the vines are staked and the fruit lifted from the ground, would seem to be far less important than under the conditions of the experiment. In the field, infection probably occurs mostly by way of the stigmas. Table 2 shows that, of the total number of fruits subjected to blown dust inoculation, 59.5 per cent became diseased, while out of the total number contaminated by painting the stigma, 79.4 per cent developed the disease. This assumption is strengthened by the experience of Martin (4) who found that the disease was spread by insects and also could be carried on the pickers' garments.

The extreme danger of infection through mechanical injury when combined with the wind process is indicated in an incident that occurred during the course of the experiment. For a few weeks of the period during which the experiment was being carried on in the greenhouse, the place was infested by a sucking white fly that punctured the fruit, stems, and leaves of the plants in order to extract the juice. The plants around a certain cluster of young flowers had been covered with newspapers in preparation for blowing the dust on the young stigmas. The ventilators of the greenhouse were open and just as the dust was being blown a gust of wind came in and spread the papers apart in line with the inoculation dust, thus exposing a cluster of fruit that had been punctured by white flies. On this cluster around each fly puncture there was alternaria infection. Although this is an isolated instance, it does seem to strengthen the evidence for wind inoculation. It should be noted that Gibson (2) reported aphid injury as contributing to the spread of alternaria infection in soy beans and cow peas. How effective wind inoculation can be is evident when compared

with the results obtained by the stab method as shown in table 2 in which 100 per cent of the fruits treated by the latter method shows infection. In other words, wind inoculation would be effective whether the spores were blown on new stigmas or on wounded fruit. Apparently, infection occurs more readily through injury than through the stigma, since 29.7 per cent of the stigmas treated did not take the infection, while mechanically injured fruit was 100 per cent susceptive. Under natural conditions, however, there is always an abundance of young stigmas subject to inoculation, while mechanical injuries are rare in comparison.

Infection of some fruit by surface contact would seem to be unimportant in the absence of accumulated moisture. Table 2 shows no infection by this method. Apparently, the fungus is unable to grow on the surface of the tomato and penetrate the stomata. This is further confirmed by the fact that, in the accident above referred to, infection from the spore-laden dust occurred only where the surface of the fruit had been punctured by sucking insects. This does not, of course, invalidate the results obtained by Douglas (1), who employed the method of surface contact by means of spores suspended in water. Where fruit of unstaked plants is allowed to lie on damp, infected ground, direct infection from resting spores or conidia might well be the main source of black rot.

The results obtained by employing these methods of inoculation, stab, painted stigma, wind, and surface contact, seem to suggest that the method of infection is primarily stigmatic, with secondary infection from mechanical injury by insects or otherwise, followed by infection-laden winds or by the actual spread of the disease by the insects as they crawl over the mechanical injuries from infected to uninfected regions.

CONCLUSION AND SUMMARY

In conclusion, it would appear that the cause of black rot in tomato is a species of Alternaria whose hyphae enter the fruit either through the stigma of a recently opened flower or through fresh wounds made by some mechanical injury, as, for example, the bite of an insect. It would appear that the infection is spread principally by winds; but the results do not preclude the transportation of the spores on the bodies and legs of insects, passing from infected to noninfected fruits.

Infection seems to occur in the fruit only, resulting in a dark brown, leathery spot, frequently occupying one-half of the tomato. This is the spot on which the conidia are produced from rapid spread of the disease. When the fruit is badly decayed so that the food becomes scarce, large resting spores are produced. Fruit, dropping to the ground, decays leaving the conidia and resting spores in the soil. The winter, if severe, kills the conidia, while the resting spores live on to be carried by the wind-blown

dust of the following season to reinfect the flowers of the new crop. In the first infected fruits conidia are again produced and are blown or carried by insects to further spread the disease.

LITERATURE CITED

1. DOUGLAS, B. New *Alternaria* spot of tomatoes in California. Phytopath. **12**: 146–148. 1922.
2. GIBSON, F. Sunburn and aphid injury of soybeans and cowpeas. Arizona Agr. Expt. Sta. Tech. Bull. 2: 41–46. 1922.
3. GUBA, E. F. Carnation blight. Massachusetts Agr. Expt. Sta. Ann. Rept. (1929/30) Bull. 271: 245–246. 1931.
4. MARTIN, W. H. Dissemination of *Septoria lycopersici* Speg. by insects and pickers. Phytopath. **8**: 365–372. 1918.
5. POOL, V. W. Some tomato fruit rots during 1907. Ann. Nebraska Agr. Expt. Sta. Ann. Rept. (1907) 21: 1–33. 1908.
6. WHETZEL, H. H. The *Alternaria* blight of potatoes in Bermuda. Phytopath. **13**: 100–103. 1923.

FUNGI OF SUGAR BEETS[1]

F. ALLEN HODGES

(Accepted for publication July 18, 1935)

INTRODUCTION

Various phases of the sugar-beet industry bring about situations in which beets are stored or piled temporarily. In seed production, beets must be stored through the winter. Including then, beets grown from seed, seedlings, maturing beets in the field, and the subsequent storing of beets, whether it be temporarily on the way to the sugar factory for slicing, or for longer periods, such as through the winter, to seed production; herein are involved many phases in which the fungal flora of beets may be of importance.

This paper comprises the study of the complete fungal flora of some 5000 beets grown at Syracuse, N. Y., and other places throughout the country.

All of the fungi found present on or in beets in the field and in storage were cultured. These include every form of fungus whether or not the form may hitherto have been considered as of economic importance. Such common forms as Penicillium, Cephalothecium, Rhizopus, and others have been given consideration.

HISTORICAL

A number of fungi already have been reported as occurring on beets, but no comprehensive list has been compiled. Duggar (2), in 1899, published his "Three important fungous diseases of the sugar beet," and Edson (4), in 1915, published a more limited work on "Seedling Diseases of the Sugar Beet and their Relation to Root-Rot and Crown-Rot." These two publications give only a meager survey of the fungi occurring on beets. Numerous forms of fungi attacking beets have been reported from time to time from this country, Europe, and Asia. Nakata *et al.* (8) have studied the diseases of beets in Korea. Seymour (10), in his "Host Index", lists various forms from beets in this country. Most of the work has been reported from Europe where the sugar-beet industry has been well-established for many years.

SOURCES OF FUNGI

The fungi recorded in this paper were secured from beets in storage, in the field, and from those grown in the greenhouse; also, from seedlings grown in the field and laboratory.

[1] Thesis, in part, submitted to the Graduate School of Syracuse University in partial fulfillment for the degree of Doctor of Philosophy.

The writer wishes to offer grateful acknowledgement to Dr. Ernest Reed, of Syracuse University, who directed this research and gave generously of his assistance and advice throughout the investigation.

A species of the genus Fusarium was of greatest frequency and was most common in stored beets, though it was secured also from field-grown seedlings, from beet seed, and from beets that had remained in the field over winter.

Alternaria occurred on all parts of the foliage of beets. It was obtained from lesions on the petioles and from leaf spots on the blade, and was found also on beets growing in the greenhouse and in the field, there producing various types of leaf spot.

Species of Rhizoctonia and Fusarium were isolated from seedlings grown in the greenhouse. Rhizoctonia was by far the most destructive of the two on the seedlings.

Phoma betae and *Sphaeropsis* sp. also were responsible for storage rots, but only to a slight degree. *P. betae* also was secured from seedlings grown in sterilized soil in the laboratory and killed by the fungus.

A few saprophytic fungi were found on beets in storage. Among these were 4 species of Penicillium, *Cephalothecium roseum*, *Acrostalagmus cinnabarinus*, and a species of Arthriniae. All of these were on the surface of the beets. Rhizopus was not noticed at first as a very destructive fungus in storage, but later was found in the inoculation experiments to be an important cause of decay.

Many species of Fusarium were obtained from sugar beets from various

FIG. 1. Localities from which species of Fusarium were obtained from beets: A. Decatur, Ind.; B. Loveland, Colo.; C. St. Louis, Mich.; D. Eaton, Colo.; E. Salt Lake City, Utah; F. Preston, Idaho; G. Betteravia, Calif.; H. Santa Ana, Calif.; I. Rocky Ford, Colo.; J. Vincennes, Ind.; K. Sterling, Colo.; L. Manteca, Calif.; M. Fremont, Ohio; N. Oxnard, Calif.; O. Spreckles, Calif.; P. Ogden, Utah; Q. Bayard, Neb.; R, S, X, and Y. Syracuse, N. Y.

sections of the United States. The States from which these forms were collected are shown on the accompanying map (fig. 1).

METHODS

Since the purpose of this work was to undertake as comprehensive a study as possible of all the forms of fungi found in connection with beets, much detailed study was given to the isolation and culture of these fungi and to their description. A considerable part of the paper, therefore, is of a taxonomic nature, dealing with a description of each form listed as follows:

Phoma betae (Fr.), *Rhizoctonia* sp., *Penicillium roseum* (Link), *P. digitatum* (Fr.) Sacc., *P. commune* Thom, *Acrostalagmus cinnabarinus* (Cda.), *Cephalothecium roseum* (Cda.), *Alternaria* sp., *Sphaeropsis* sp., *Arthriniae* sp., *Mucor heterosporus* (Fisher), *Rhizopus nigricans* (Ehr.), *Cylindrocarpon radicicola* (Wr.) var. *violaceum* Hochapfel ad int., *C. didymum* (Hart.) Wr., *C. radicicola* Wr., *Fusarium arcuatum* Berk. et Curt., *F. arcuosporum* Sherb., *F. bulbigenum* Cke. et Mass., *F. bullatum* var. *minus* Sherb., *F. chenopodium* (Thuem) Sacc., *F. clavatum* Sherb., *F. culmorum* (W. G. Sm.) Sacc., *F. dimerum* Penz., *F. dimerum* Penz. var. *pusillum* Wr., *F. discolor* App. et Wr., *F. diversisporum* Sherb., *F. equiseti* (Cda.) Sacc. var. *bullatum* (Sherb.) Wr., *F. equiseti* (Cda.) Sacc. var. *crassum* Wr., *F. ferruginosum* Sherb., *F. herbarum* (Cda.) Fries, *F. heterosporum* Nees, *F. lateritium* Nees, *F. lutulatum* Sherb., *F. martii* App. et Wr., *F. merismoides* Cda., *F. merismoides* Cda. var. *majus* Wr., *F. moniliforme* Sheldon, *F. orthoceras* App. et Wr., *F. orthoceras* App. et Wr. var. *albido-violaceum* (Daz.) Wr., *F. orthoceras* App. et Wr. var. *longius* (Sherb.) Wr., *F. orthoceras* App. et Wr. var. *triseptatum* Wr., *F. oxysporum* Schl., *F. radicicola* Wr., *F. redolens* Wr., *F. sambucinum* Fckl., *F. scirpi* Lamb. et Fautr., *F. scirpi* Lamb. et Fautr. var. *acuminatum* (Ell. et Ev.) Wr., *F. scirpi* Lamb. et Fautr. var. *caudatum* Wr., *F. scirpi* Lamb. et Fautr. var. *compactum* Wr., *F. scirpi* Lamb. et Fautr. var. *filiferum* (Preuss) Wr., *F. semitectum* Berk. et Rav., *F. semitectum* Berk. et Rav. var. *majus* Wr., *F. solani* (Mart.) App. et Wr., *F. solani* (Mart.) App. et Wr. var. *martii* (App. et Wr.) Wr., *F. solani* (Mart.) App. et Wr. var. *medium* Wr., F. *solani* (Mart.) App. et Wr. var. *minus* Wr., *F. solani* (Mart.) App. et Wr. var. *suffuscum* Sherb., *F. sporotrichioides* Sherb., *F. subulatum* App. et Wr., *F. trichothecioides* Wr., *F. truncatum* Sherb., *F. ventricosum* App. et Wr., *F. viride* (Lechm.) Wr., *F. flocciferum* Cda., *F. reticulatum* Mont.

In the cultural studies several types of media were used. It was thought that the different media would provide possibilities for fungus variations that might occur in response to the nutriment furnished. No variations,

however, were observed and any one of the several media would have served the purposes fully as well as any other. The media used were:

1. Synthetic (maltose 6 grms. per liter) (6), 2. Edson (dextrose 100 grms. per liter) (4), 3. Cornmeal (3), 4. Red beet (3), 5. White beet (3), 6. Rice.

The identification of the species and varieties of Fusarium was determined chiefly through the work of Wollenweber's (13) "Fusaria Autographice Delineata." Other sources were consulted and include the following:

Appel and Wollenweber (1), Wollenweber (12–14), Sherbakoff (11), Reinking and Wollenweber (9), Wollenweber *et al.* (15), Link and Bailey (7).

A number of the cultures were sent to Dr. Wollenweber for identification.

Inoculation studies were made in which the matured beet under storage conditions was used, and the effect of inoculations on growing beets in the seedling stage was observed.

BEETS IN STORAGE

Three sets of cultures were set up. Two of these consisted of the inoculation of beet roots, placing them in boxes of moist sand, and storing them in the laboratory at room temperature (21° C.).

For these experiments the beets first were washed in water to remove the dirt. They were then washed in a 1:1000 solution of mercuric chloride and allowed to dry. In order to introduce the fungus into the beet, a hole was made completely through the latter near the crown by means of a 5 mm. cork borer. Another device used to make a hole through the beet was a $\frac{1}{4}$ inch bit attached to an electric motor.

Stock cultures of the fungi served as inoculum. Pieces of the mycelium, masses of spores, sporodochia, or pionnotes, as the case might be were placed in the hole of the beet, and the ends of the hole were plugged with sterilized cotton. The beets were labeled and packed in moist sand. Figure 2, C, represents a noninoculated beet, used as a control.

In the first set of experiments 3 beets were inoculated with each fungus. The beets thus inoculated were labeled and packed in boxes of sand. At the end of a period of about 4 weeks, the beets were removed and examined. In some instances it was observed that the fungi produced excellent symptoms of rot, while in others no penetration took place. Other beets were so rotted by fungi that were not used as inoculum that results were unsatisfactory. There also was loss of material due to destruction of the labels.

The second set of experiments was conducted similarly, but only those fungi were used that gave unsatisfactory and inconclusive results in the

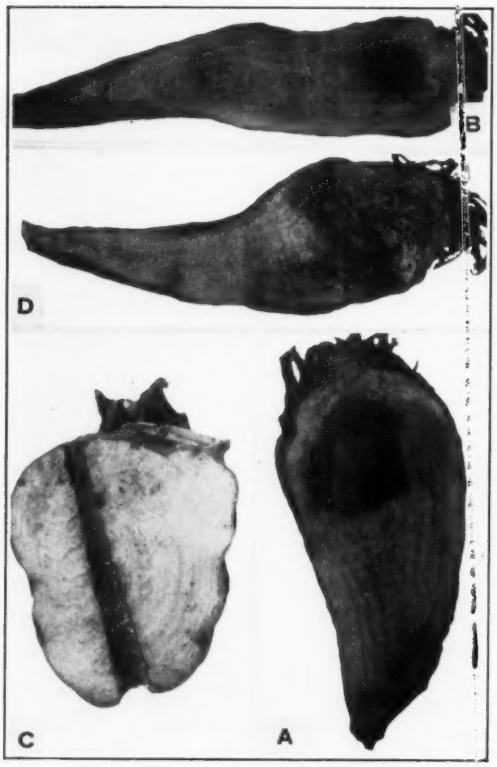


FIG. 2. A. Inoculated beet showing the rot produced by *Fusarium orthoceras*. B. Beet inoculated with the fungus *F. dimerum*. C. Sugar beet used as a control, shows the hole through the beet for inoculation purposes. D. Inoculated beet, showing the rot produced by the fungus *F. ventricosum*.

first set. For this experiment 24 beets were inoculated with 8 fungi and placed in a single box of sand. By employing this method the boxes were not overcrowded and the beets could be more evenly spaced. They were prepared and inoculated in the same manner as in the preceding experiment.

The boxes were stored in the laboratory, but the beets were not left in the sand so long as were those of the first set of experiments. At the end of 2 weeks, the beets were removed and examined. It was found, however, that the results were even less satisfactory. There was considerable loss of material due to contamination by bacteria and other fungi. However, some of the beets were unaffected by contaminations and showed good results from the inoculations.

Although the results obtained in these two experiments were not fully satisfactory, yet the amount of damage done to the beets that were rotted by some of the fungi, indicated the importance of the latter as rot-producing

organisms of stored beets. Those forms that were considered most active in producing rots and, therefore, the most destructive, are listed below.

Phoma betae, Rhizoctonia sp., *Sphaeropsis* sp., *Fusarium dimerum, F. orthoceras, F. semitectum* v. *majus, F. ventricosum, F. diversisporum, F. arcuosporum, F. merismoides, F. solani, F. chenopodium, F. clavatum, F. bullatum* v. *minus, F. trichothecioides, F. radicicola, F. scirpi* v. *filiferum.*

Some of the rots produced in these stored beets by a few of the species of Fusarium listed above are shown in figure 2, A, B, and D.

The third set of experiments consisted in placing the inoculated beets in damp chambers instead of storing them in boxes of sand. This method was accomplished by putting a single inoculated beet into a quart Mason jar. One beet was used for each fungus. All of the jars were sterilized in the autoclave and tap water used; the water changed every day during the course of the experiment, with just enough to cover the bottom of the jar.

The beets were washed, sterilized, and inoculated as in the preceding experiments. The jars were covered with a piece of cotton and the jar tops inverted on them.

The sets of inoculations were run for a period of 7 days. The beets were then removed and the amount of penetration by each fungus was determined by carefully cutting through the beet at the place of inoculation.

The fungi varied in their ability to penetrate the beet tissue. Some were rapid growers and produced excellent penetration, even to enlarging the hole through the beet. Others showed no sign of penetration in contrast with the cultures of the first experiments. In cases where contaminating fungi were found attacking the surface of the beet, the infected part was cut off to check any further development of the lesions. Many of the beets induced the growth of thick, matted roots and leaves.

This method of inoculation seemed the most satisfactory in that it showed whether the fungus was capable of penetrating the tissues of the growing host, and of entering the turgid cells. It, therefore, afforded a better idea as to the real pathogenicity of the fungi than did the other experiments, and it also offered less opportunity to contaminating forms.

In addition to fungi listed above, the following species of Penicillium, Rhizopus and Fusarium may be considered as important causes of storage rots of the beet: *Penicillium commune, Rhizopus nigricans, Fusarium culmorum, F. arcuatum, F. discolor, F. dimerum* v. *pusillum.*

As in the other experiments, the fungus *Fusarium dimerum* was found to produce the most damage to beets in the damp chamber.

The results obtained from these experiments involving inoculation of beet roots would indicate that some of the fungi are much more destructive than others, while some are quite innocuous. Especially in the case of the damp-chamber cultures, where the beets were growing to a slight degree,

penetration by the fungus was more significant in that a good penetration would indicate a strong pathogenic nature of the fungus. Where no, or only a slight, penetration was obtained, the fungus would be of lesser importance and cause only a slight loss of beets in storage.

Fusarium dimerum and *F. orthoceras* appeared to be most actively parasitic in that they produced the most extensive penetration in the roots in all of the experiments.

Of the 85 species and varieties of fungi used in these experiments, only 23 showed any appreciable effect on stored beets and some of these produced only slight penetrations.

SEEDLING INOCULATION EXPERIMENTS

Another phase of the experimental study of the fungi of beets, was that made to find out what effect, if any, such fungi would produce on the growing plant. To this end seedlings were grown in the laboratory and in the field.

Three-inch flower pots of soil were sterilized in the autoclave at 15 pounds pressure for 1 hour on 2 successive days. Seeds of the variety Menomonee were planted in the pots, and before planting, the seeds were rinsed in a 1:1000 solution of mercuric chloride, followed by rinsing in distilled water. From 10 to 15 seeds were planted in each crock and the crocks kept in the laboratory at room temperature, covered with sterilized Petri dish covers.

As soon as the seedlings showed above the surface of the soil, spore suspensions, in boiled tap water, were made from each fungus. These suspensions were made by filling a test tube (about 25 cc. capacity) with the boiled tap water and putting into it masses of mycelial tissue. The tube was then shaken thoroughly. One tube of these spore suspensions was made for each fungus used and it was poured over and around the seedlings in a crock. One crock was used for each fungus. At the end of about a week the seedlings were examined to note any effect the fungi may have had upon them. Those seedlings that showed any injury were carefully removed from the flower pots, washed in distilled water to remove the dirt, dipped in mercuric chloride (1:1000), rinsed in distilled water, and placed in Petri dishes containing the synthetic medium (6). On development of the fungus in culture, it was identified and its characteristics were studied.

The results of this study showed that only about 20 per cent of the fungi employed produced positive results and were capable of causing seedling infection. From this it would appear that the fungi have, in most cases, little, if any, effect on beet seedlings and need not be considered as important pathogens of the growing beet.

As was found in the previous inoculation experiments, *Fusarium*

dimerum and *F. orthoceras* proved to be the most active parasitic forms. They produced from 50 to 75 per cent infection in the seedlings.

Inoculation studies of seedlings grown in the field were made chiefly with the numerous forms of the genus Fusarium obtained from various parts of the country. (Fig. 1). Details of the results obtained will be considered later. Suffice it to be stated here that, in general, the results were negative and only a few species attacked the seedlings. Of these, *Fusarium orthoceras* was as active a parasite under field conditions as on beets in storage.

TYPES OF ROTS PRODUCED BY THE FUNGI IN STORED BEETS

During the course of the investigations on inoculation of beets in storage, it was noticed that the fungi used produced different types of rot.

Three such rots were noted: (1) A distinctly dry, corky rot, producing in some cases enlargement of the hole through the beet. This enlargement

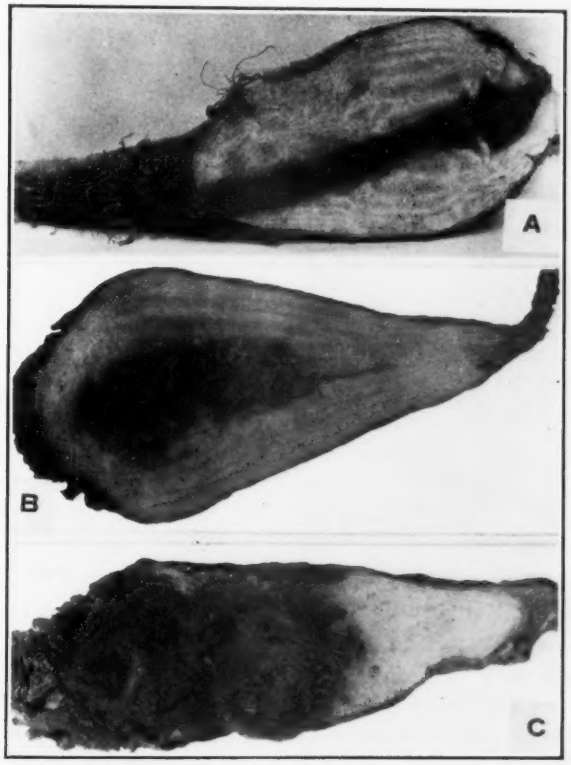


FIG. 3. A. Dry, corky rot produced by *Phoma betae*. B. Soft, watery rot also produced by *P. betae*. C. Intermediate type of rot produced by the fungus *Fusarium orthoceras*.

was of different degrees in the various cases noted. Fig. 3, A, shows such a rot produced by *Phoma betae*. (2) A soft, slimy type that produced a wet, water-soaked appearance in the beet. Fig. 3, B, shows this type and it also was produced by the fungus *Phoma betae*. (3) A third type was one that was neither a corky nor a wet rot. The fungus penetrated the substratum, destroyed the tissue, produced a type of rot that could not be designated either as a dry, corky rot nor yet as a wet rot. It seemed to be a type in which many cavities were formed, and in some cases these cavities were filled with a mycelial growth. This was designated as an intermediate rot. Fig. 3, C, shows this type produced by *Fusarium orthoceras*.

These observations were recorded at the time the beets were examined to study the effects produced by the inoculations with the various fungi. This represented merely additional data to the inoculation studies and no further detailed study was given these observations.

GENERAL DISCUSSION

The material used in this investigation was secured from sugar beets grown at Syracuse, N. Y., and in various other parts of the United States. The distribution of the localities which supplied material is shown in figure 1. The fungi used in this study have been secured from beets grown in the field, from beets in storage, and from beet seedlings grown in the field, laboratory, and greenhouse. Beet seed obtained from Europe was the source of some of the fungi studied.

Only a few forms of those fungi previously mentioned in the literature were found during the progress of these studies. These are as follows:

Phoma betae, *Rhizoctonia* sp., *Alternaria* sp., *Rhizopus nigricans*, *Pionnotes betae*, *Fusicola betae*, *Cephalothecium roseum*, *Fusarium argillaceum*, *F. reticulatum*, *F. ventricosum*, *F. merismoides* v. *majus*.

The following forms: *Fusarium argillaceum*, *Fusicola betae*, and *Pionnotes betae*, mentioned by others, may all now be considered as species of the genus Fusarium. Wollenweber (14) reports only a few Fusarium species found on beets. He states that the name *F. betae*, which is synonomous to Saccardo's *Pionnotes betae*, is now changed to *Fusarium merismoides* v. *majus*. In his *Fusaria Autographice Delineata* (13) he states that *F. betae* is a synonym for *Cercospora beticola* (Sacc.), which is reported as attacking the foliage of beets. *Fusisporum argillaceum* (Fries.) he identifies as *Fusarium argillaceum* (Fr.) Sacc. and to which he gives the synonomous name *Fusarium ventricosum* App. et Wr.

The following fungi comprise those here reported for the first time as prevalent on the beet:

Penicillium roseum, *P. digitatum*, *P. commune*, *Acrostalagmus cinna-*

barinus, *Sphaeropsis* sp., *Arthriniae* sp., *Mucor heterosporus*, *Cephalosporium* sp., *Spicaria griseola*, *Haplosporella* sp., *Cylindrocarpon radicicola*, *C. radicicola* v. *violaceum*, *C. didymum*, and *Fusarium* sp. (about 49 spp.) and var. as listed on page 561.

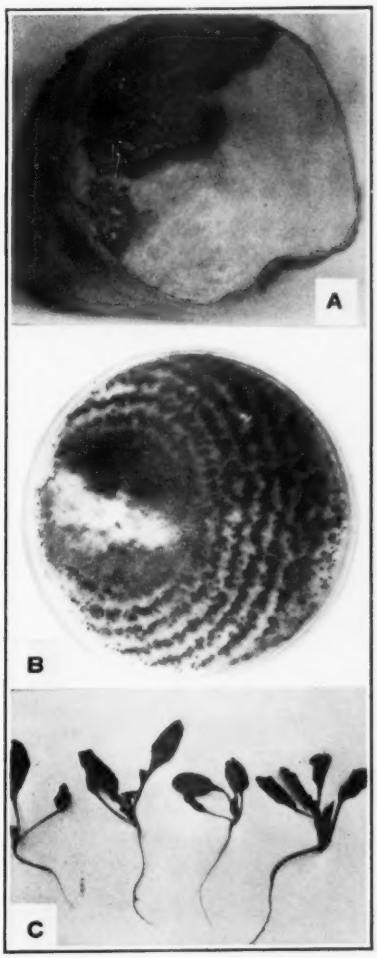


FIG. 4. A. Rot produced by *Phoma betae* in a stored sugar beet. B. Culture of *P. betae* on corn-meal medium, showing the production of pyenidia. C. Root-rot of sugar beet seedlings produced by the fungus *Rhizoctonia* sp.

Though *Phoma betae* often has been reported as a destructive fungus of beets and has been mentioned recently by Grooshevoy (5) as the cause of a serious root rot of beet seedlings, it was not found to be so prevalent in this investigation (Fig. 4).

Cercospora beticola causes widespread defoliation of beets in certain sections of this country and Europe, yet it was not abundant under observation in this study. This may be due to the fact that the summers of this locality are cooler than in those areas where Cercospora is so prevalent.

Alternaria sp. was found as a leaf spot in this locality. The damage done by Alternaria does not seem to be so vital as that caused by Cercospora, since it does not defoliate beets, whereas Cercospora has been known to do so, 2 and even 3 times in a season. Various types of leaf-spot infection and petiole lesion were found attributable to this species of Alternaria, hence it was regarded as a parasite and was found to cause a slight defoliation of beets growing in the field.

Beet rust, beet mosaic, and bacterial diseases of beets were not observed in this study. The beet nematode, *Heterodera schactii*, presents a real problem for the beet growers in some of our western States, but not in this region.

Though *Rhizoctonia* sp. produced some decay in stored beets, it was more destructive of seedlings. (Fig. 4, C.)

The genus Fusarium has been considered of no very great importance in beet culture; but, in the light of these studies, it appears that Fusaria probably cause the largest number of rots in beets. The number of species of Fusarium secured from beets was large. These have been studied on the assumption that they may all differ in some respects. The data have justified this assumption, for it will be noted that certain Fusaria that appear taxonomically identical are not alike in other respects. Many duplicate cultures were, therefore, maintained and each treated as a separate and distinct species. The various species collected and studied have been shown to be more important as storage-rot fungi, and only a very few proved to be parasitic on the beet seedlings. There were certain forms, however, that had little or no effect on stored beets.

In the studies made on the inoculation of beets in storage, it was observed that different types of rot were produced by the fungi used. The results indicated that a few of the forms were capable of producing all types of rot, namely; a dry, corky rot; a wet, slimy rot; and what was termed an intermediate rot. Other forms produced only one type. This phase of the work, however, was merely suggestive and is presented in the form of additional data. Conclusions are withheld pending further observations.

Referring again to those fungi considered in this paper and listed on page 561 the question arises as to which of these forms are parasitic and which are merely secondary invaders. Those forms attacking the growing beet and those causing considerable decay of beets in storage are considered as parasitic fungi. The secondary invaders may be included in

TABLE 1.—*Fungi isolated from beets and arranged according to their economic importance*

Group A. Parasitic fungi			Group B. Saprophytic fungi (stored beets)	
Beets in storage	Seedlings in field	Seedlings in laboratory	Cause little or no decay	Of no economic importance
Phoma betae	*Fusarium orthoceras*	*Rhizoctonia* sp.	*Cylindrocarpon radicicola* var. *violaceum*	*Penicillium funiculosum*
Rhizoctonia sp.	*Fusarium solani*	*Penicillium roseum*	" *radicicola*	*Acrostalagmus cinnabarinus*
Alternaria sp.	*Fusarium merismoides*	" *digitatum*	*Fusarium arcuosporum*	*Mucor heterosporus*
Sphaeropsis sp.		*Rhizopus nigricans*	" *bulbigenum*	*Cylindrocarpon didymum*
Penicillium commune		*Fusarium orthoceras*	" *equiseti* v. *crassum*	*Cephalothecium roseum*
Rhizopus nigricans		" *equiseti* v. *bullatum*	" *ferruginosum*	
Fusarium arcuatum		" *dimerum*	" *herbarum*	
" *bullatum* v. *minus*		" *scirpi* v. *filiferum*	" *heterosporum*	
" *chenopodium*		" *solani*	" *lateritium*	
" *clavatum*		" *lutulatum*	" *merismoides* v. *majus*	
" *culmorum*			" *moniliforme*	
" *dimerum*			" *orthoceras* v. *longius*	
" *dimerum* v. *pusillum*			" *orthoceras* v. *triseptatum*	
" *discolor*			" *oxysporum*	
" *diversisporum*			" *redolens*	
" *merismoides*			" *sambucinum*	
" *orthoceras*			" *scirpi*	
" *radicicola*			" " v. *caudatum*	
" *semitectum* v. *majus*			" " v. *acuminatum*	
" *trichothecioides*			" *sclerotioides*	
" *ventricosum*			" *semitectum*	
			" *solani* v. *martii*	
			" " v. *suffuscum*	
			" *sporotrichioides*	
			" *subulatum*	
			" *truncatum*	
			Several unidentified *Fusaria* spp.	

the list of the saprophytic fungi, which, while they occur on beets in storage, may not be decay producing organisms. From this point of view, therefore, an attempt has been made to provide a list of those fungi that might be considered of economic importance to the sugar beet industry and those that are of little or no importance. Table 1 presents a summary of these forms from this economic point of view. Those forms that fall under group A—parasitic fungi—may be considered as those that would be most destructive to beets both in storage and as seedlings; while those of group B—the saprophytic fungi—may be considered as of less importance and productive of little injury or loss under storage conditions.

Since the field-inoculation studies produced, on the whole, negative results, with the exception of those fungi listed in table 1, group A, the majority of the fungi would not be considered as serious parasites of the growing beet.

SUMMARY

About 5000 beets were examined for the presence of fungi. This included beets grown in the field and greenhouse, beets in storage, matured beets, and beet seedlings, and beets grown near Syracuse, N. Y., and from various sections of the United States.

Pure cultures were made of each of the fungi isolated.

The fungi thus isolated were divided into groups as follows:

A. *Parasitic fungi*
   1. those of storage importance
   2. those attacking the beet in the seedling stage

B. *Saprophytic fungi*
   1. those producing little or no decay in stored beets
   2. those of no economic importance, or the so-called secondary invaders.

Species of the genus Fusarium were responsible for most of the storage rots.

A number of fungi, including a great many species of Fusarium, are reported on beets for the first time.

Alternaria sp. is here reported for the first time as a parasite of beets in New York State.

Identification and description of about 50 species and varieties of the genus Fusarium are reported.

The role of *Phoma betae* as a parasite of beets is of minor importance among the fungi under these studies.

The parasitic importance of many of the species of Fusarium on growing beets has been shown to be negligible, and of little economic significance to the sugar beet industry.

Only a few of the fungi studied appeared to be active parasites and may be considered as important plant pathogens.

SYRACUSE UNIVERSITY
SYRACUSE, N. Y.

LITERATURE CITED

1. APPEL, O., AND H. W. WOLLENWEBER. Grundlagen einer Monographie der Gattung *Fusarium* (Link). Arb. Biol. Anst. Land. u. Forstw. **8**: 1–207. 1910.
2. DUGGAR, B. M. Three important fungus diseases of the sugar beet. New York (Cornell) Agr. Expt. Sta. Bull. 163. 1899.
3. ————. Fungous diseases of plants. 508 pp. Ginn & Co. Boston, New York [etc.]. 1909.
4. EDSON, H. A. Seedling diseases of the sugar beets and their relation to root-rot and crown-rot. Jour. Agr. Research [U. S.] **4**: 135–168. 1915.
5. GROOSHEVOY, S. E. Root rot disease of the seedlings of sugar beets. Proc. Exper. Select. Stat. Mironovka Kieff. Pp. 1–49. 1931. [In Russian. English summary. Abstract in Rev. Appl. Mycol. **11**: 760. 1932.]
6. LEONIAN, L. H. A study of factors promoting pycnidium formation in some *Sphaeropsidales*. Amer. Jour. Bot. **11**: 19–50. 1924.
7. LINK, G. K .K., AND A. A. BAILEY. *Fusaria* causing bulb rot of onions. Jour. Agr. Research [U. S.] **33**: 929–952. 1926.
8. NAKATA, K., T. NAKAJIMA AND S. TAKIMOTO. Studies on sugar beet diseases and their control. Chosen. Agr. Expt. Sta. Bull. 6. 1922. [In Japanese. Abstract in Japan. Jour. Bot. **1**: (43). 1923.]
9. REINKING, O. A., AND H. W. WOLLENWEBER. Tropical *Fusaria*. Philippine Jour. of Sci. **32**: 103–253. 1927.
10. SEYMOUR, A. B., comp. Host index of *Fungi* of North America. 732 pp. Harvard University Press. Cambridge, Mass. 1929.
11. SHERBAKOFF, C. D. *Fusaria* of potatoes. New York (Cornell) Agr. Expt. Sta. Mem. 6. 1915.
12. WOLLENWEBER, H. W. Studies on the *Fusarium* problem. Phytopath. **3**: 24–50. 1913.
13. ————. Fusaria autographice delineata. 56 pp. 1100 plates. R. Friedländer & Sohn. Berlin. 1916–1930. [Text reprinted from Ann. Mycol. **15**: 1–56. 1917.]
14. ————. Fusarium-monographie. Fungi parasitici et saprophytici. Ztschr. Parasitenk. **3**: 269–516. 1931.
15. ————, C. D. SHERBAKOFF AND O. A. REINKING. Fundamentals for taxonomic studies of *Fusarium*. Jour. Agr. Research [U. S.] **30**: 833–843. 1925.

A STUDY OF THE TOXIC ACTION ON GRAY-MOLD SPORES OF CLEANING SOLUTIONS USED IN SPRAY RESIDUE REMOVAL[1]

OTTO F. SCHNELLHARDT[2] AND F. D. HEALD

(Accepted for publication June 19, 1935)

INTRODUCTION

Apples, produced in the central irrigated valleys of Yakima and Wenatchee, are practically free from either decay or fungous lesions at the time of harvest, but storage diseases take a heavy toll of the harvested crop each year. Heald and Ruehle (8) recorded more than 40 species of fungi belonging to 22 genera as connected with decay of apples in Washington; of these blue mold and gray mold were probably the most destructive. Brien (3) in New Zealand found gray mold (*Botrytis cinerea*) causing an average of 16.83 per cent infection as compared to 5.6 per cent for *Penicillium expansum* on apples in cool storage. Examination[3] of boxed apples in the Wenatchee area during the 1933–34 storage season indicate that gray mold may become destructive as a rot of stored apples wherever there is considerable rainfall during the harvesting period. Gray mold advances more rapidly in cold storage than blue mold. Fruit inoculated with pure cultures of the fungus may be completely rotted after two months at 0° C. or after two weeks at room temperature. Botrytis of the cinerea type is the most important gray-mold species causing decay of apples in Washington.

The fruit cleaning methods now in use consist of washing the fruit with various solvents. Hydrochloric acid or some of the alkaline compounds are the most common cleaners. These solutions are ordinarily heated in commercial practice to temperatures of 90° F. to 120° F. and occasionally as high as 130° F. The higher temperatures, however, often cause injury to certain varieties, either bleaching the skin or lowering the keeping qualities. During the last two seasons the types of washers previously used are being abandoned and new types adopted, largely on account of the use of tandem or double washes. When tandem washes are employed to clean fruit sprayed with lead arsenate, excellent cleaning may result with either the sequence of first, HCl, and second, sodium silicate, or the reverse. Other

[1] Published with the approval of the Director of the Washington Agricultural Experiment Station as Scientific Paper No. 314, College of Agriculture and Agricultural Experiment Station, State College of Washington, Pullman, Washington.

[2] Formerly Research Assistant in Plant Pathology, Washington Agricultural Experiment Station, Pullman, Washington.

[3] Data collected by Mr. G. A. Newton while working on the apple rot survey in Washington.

alkaline cleaners, such as sodium carbonate-trisodium phosphate or soda ash, may be substituted for the sodium silicate when a milder alkali is desired.

Constant changes in methods and materials used in apple washing leads one to wonder what effect these alterations are having on the life of the fungous spores present in the washing and rinse tanks. Fruit-washing tanks have been found by various investigators to be a source of contamination by fungous spores. Heald *et al.* (7) in Washington, Pentzer in New York (10), Shear in the Hudson Valley (12), and Fisher and Reeves (6) found an accumulation of fungous spores in the cleaning and rinsing baths during commercial operations. The solvents used and the temperatures of the cleaning solutions in the earlier washing operations had little or no killing effect on the spores. Diehl *et al.* (5) stated certain rot-producing spores were able to survive exposure to either acid or alkaline washing solutions for 6 days. These spores originated from the surface of normal apples, from an occasional decayed apple that passed into the machine, and from the dust of the air.

In recent work Baker and Heald (1) have shown that temperatures of washing solutions within the ranges employed, exert a lethal effect on the spores of *Penicillium expansum*. The killing effect was markedly increased by addition of the washing compound. These two investigators (2) have further shown that treating the apples with a sodium hypochlorite rinse, following washing, is very effective in reducing the spore load of the surface of the fruit and the lenticel basins. It further decreased the percentage of decay at punctures and other points of entry.

MATERIALS AND METHODS

In this study on the toxic action of cleaning solutions the cultures used were transfers from the Botrytis isolated from decayed apples by Heald and Ruehle (8) and identified by them as *Botrytis cinerea* Pers. The conidia used were taken from vigorously growing 10 to 15-day-old colonies cultured on 2 per cent dextrose-potato agar.

The cleaners tried out experimentally in this work were those most commonly used in the packing plants during the 1933 and 1934 seasons. Hydrochloric acid at a strength of 3 per cent by volume was tested for the acid cleaner. In terms of this experiment it was 9 cc. per 300 cc. water blank. Two alkali cleaners were tested. The first of these was sodium carbonate at a concentration of 75 pounds per 100 gallons of water or 26.96 grams per 300 cc. water blank. This product was called Wyandotte soda ash, manufactured by the Michigan Alkali Company, Wyandotte, Michigan. The second alkali cleaner was sodium silicate used at the same concentration. This is called "B. W." silicate of soda and is manufactured by the Philadelphia Quartz Company of California, Ltd., Berkeley, California.

The methods employed were similar to those used by Baker and Heald

(1) with modifications better adapted for work with the gray-mold organism. In the tests with commercial hydrochloric acid as a cleaner, spores were scraped from Petri dish cultures and placed in a flask containing 100 cc. of spore-free water and shaken for 5 minutes to break up the spore clumps. Ten cc. of this concentrated spore suspension was transferred to flasks in triplicate containing 300 cc. sterile distilled water plus 9 cc. of the commercial HCl. A similar flask of water containing a spore suspension without the HCl was used as a check. These flasks were thoroughly agitated and 1 cc. from each was transferred with a sterile pipette to 9 cc. sterile water blanks, making a 1–10 dilution. Tests were made from these dilutions at timed intervals and controlled temperatures. The dilutions were placed on sterile Petri dishes with sterile pipettes and 2 per cent dextrose-potato agar cooled to about 42° C. poured over them and the dishes rotated. After 48 hours' incubation at room temperature the number of colonies per plate was counted, and an average for the three plates taken. From this count the number of gray-mold spores per cubic centimenter was calculated. Thus the figures (Table 2) are averages of the 3 plates made from each flask at each viability test and are expressions of the approximate number of viable gray-mold spores per cubic centimeter in the solution at the time the test was taken.

In the sodium carbonate tests (Table 3) the spore concentration was increased by transferring 20 cc. of the suspension to the flasks instead of 10 cc. Three of the flasks each contained 26.96 grams of soda ash plus 300 cc. sterile distilled water. One flask contained no washing compound and was used as a check. The dilutions were plated out in duplicate and an average of the 2 plates taken to determine the number of viable spores per cubic centimeter. The other procedure was the same as for the HCl tests.

The sodium silicate cleaner exhibited such a marked toxicity to the Botrytis spores that fewer tests were made, and the time intervals were shortened (Table 4). This required a rapid transfer from the flasks to the dilution tubes when the cleaners were tested at the higher temperatures. Only 2 flasks were used in this series. One contained 26.96 grams of sodium silicate, and the other without the cleaner was used as a check. These were tested at one-half and one minute intervals.

The effect of these cleaners on spore survival was tested at room temperature (68–70° F.), 90° F., 100° F., 110° F., and 120° F. In order to obtain the effect of the solutions at different temperatures the flasks were placed in an incubator. The flasks were placed in pans of water while being held in the incubator to stabilize the temperature and to reduce the evaporation of the solutions that would have increased the concentration of the treating compound.

The temperatures of the flasks were stabilized by keeping them in an incubator overnight at the desired degree, after which the spores were added. Transfers from the flasks were made in a sterile culture chamber and performed as quickly as possible. During this time the flasks were held in the water pans and the temperature fluctuation was slight.

Commercial apple-washing practices were duplicated as nearly as possible in these laboratory tests. The spores in suspension in the flasks were subjected to both the lethal effect of the solute and to that of the temperature. This is similar to the conditions in the packing plant, where the fungous spores are in a heated washing tank in the presence of a cleaner. Thus, the results obtained would give some indication of the toxicity of these cleaners at the temperatures employed. These in turn could be used as indices to determine the lethal point of gray-mold spores. Spore viability was determined by the ability to form colonies on 2 per cent dextrose-potato agar plates.

The degree of experimental variation was determined (Table 1). Four flasks of 300 cc. sterile distilled water were inoculated with a spore suspension and 10 plates poured at once from each flask, using 1 cc. of a 1–10 dilution. At times the range of experimental variation appeared fairly broad, but this probably was due to the variable number of viable spores transferred with the 1 cc. pipette to the 9 cc. water blank.

The subsequent transfer of the dilutions to the sterile Petri dishes would again vary somewhat because the number of spores in each dilution would show some deviation. The average of the plates poured, within certain limits, showed considerable uniformity of results.

TABLE 1.—*Possible experimental variation of viable spores of Botrytis per cubic centimeter from the same spore suspensions*

Plate[a]	Flask 1	Flask 2	Flask 3	Flask 4
1	1370	1230	780	970
2	1270	1540	1030	1280
3	1120	1470	970	1170
4	1060	1280	1000	1480
5	1370	1620	1220	1330
6	1070	1560	1010	1350
7	1030	1580	900	1190
8	980	1330	960	1050
9	1340	1530	820	1170
10	1330	1420	1040	1280
Ave.	1194	1456	973	1227

[a] One cc. of spore suspensions placed in 9 cc. sterile water blanks from which 1 cc. was removed to each poured plate.

THE EFFECT OF CLEANING SOLUTIONS ON GRAY-MOLD SPORES AT DIFFERENT TEMPERATURES

The effect of hydrochloric acid on gray-mold spores at the usual strength of commercial cleaning and at various temperatures was determined (Table 2). This cleaner exerted a marked toxic effect on gray-mold spores, which increased with increased temperature and longer exposures. At room temperature 81.3 per cent of the spores were killed at the end of 104 hours with no reduction occurring in the water check after this time. The reduction in number of gray-mold colonies resulting from the action of the cleaner after 72 hours at room temperature is shown in (Fig. 1, A, D). At 90° F. in the water check, 55.6 per cent of the spores were killed after 60 hours, while in the cleaner they were all dead after 25–36 hours. At 100° F. the water check showed a 54.8 per cent kill after 24 hours and a complete kill after 7 hours in the cleaner. The spores were all killed after 12 hours' exposure in the water check at 100° F. and viability ceased after 2 hours in the cleaner. At 120° F. all spores were killed after 35 minutes in the water check and the lethal point was reached in 15 minutes with the addition of the cleaner.

The sodium carbonate cleaner (Table 3) showed a marked effect on the Botrytis spores at 90° F. and at room temperature. A 100 per cent kill and a 98.8 per cent kill, respectively, took place at these two temperatures after 101 hours, while the water checks showed no reduction in number of colonies. At 100° F. the soda ash gave a kill of 99.6 per cent as compared to 52.2 per cent for the water check after 36 hours, while at the end of 48 hours viability ceased in both the water check and the cleaner. The reduction in number of colonies on account of the soda ash treatment at 100° F. is well shown in the illustration (Fig. 1, B, E). When subjected to a temperature of 110° F. for 11 hours, the spores were killed in both the water check and the cleaner, while in the cleaner there was a nearly complete kill after 7 hours. In flasks held at 120° F. the spores were all killed in 20 minutes as contrasted to 30 minutes for the water check.

Sodium silicate had the greatest effect on the viability of gray-mold spores. No reduction was noticeable in spore number in the water checks of this series because of the short time the spores were held therein. The flasks of cleaner showed a greater reduction in spore number as temperature and time of exposure were increased. The results (Table 4) show a 95.5 per cent kill at room temperature after 5 minutes; a complete kill at 90° F. after 5 minutes; all spores dead after 3 minutes at 100° F.; no viable spores after 2 minutes at 110° F.; and a complete kill in 1 minute at 120° F. Reduction in colony number by exposure at 90° F. for 3 minutes is shown in figure 1, C, F.

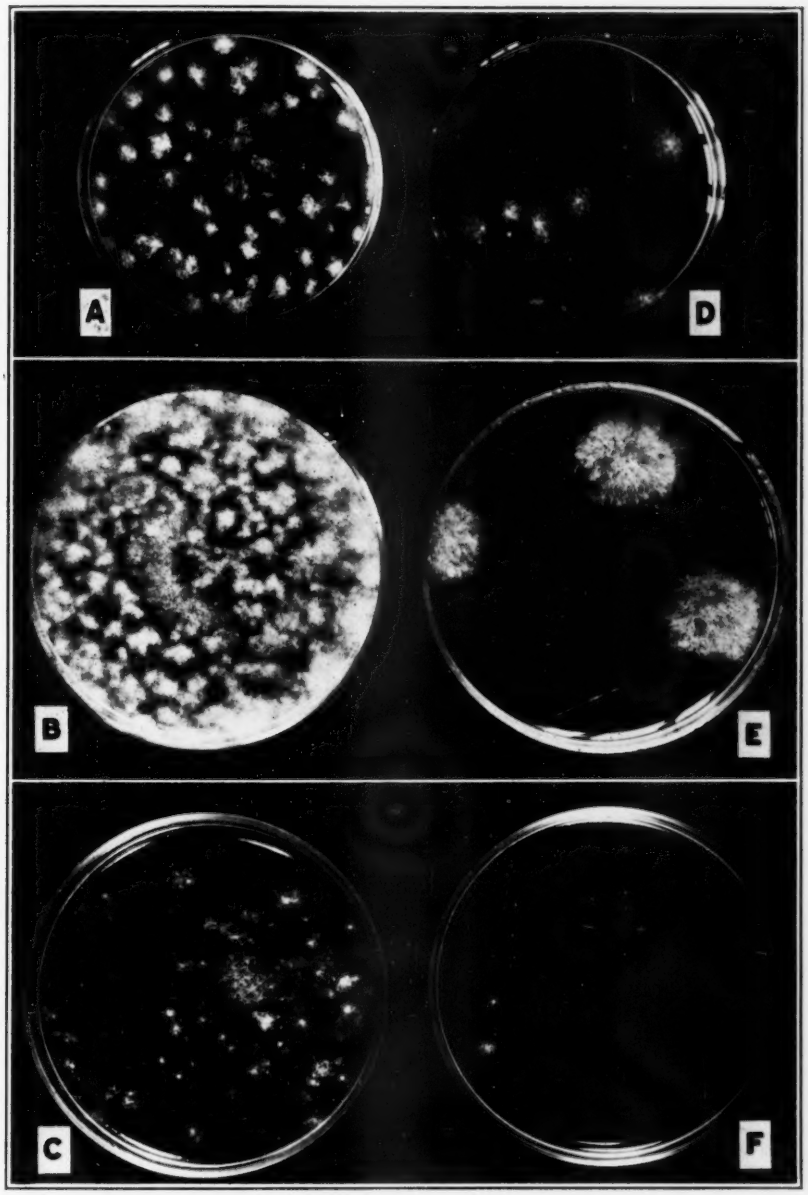


FIG. 1. Colonies of *Botrytis cinerea* on 2 per cent potato-dextrose agar. A–C. Water checks. D. From flask held 72 hours at room temperature in 1 per cent HCl. E. From flask held 30 hours at 100° F. in soda ash cleaner. F. From flask held 3 minutes at 90° F. in sodium silicate cleaner.

TABLE 2. *Number of viable gray-mold spores per cubic centimeter obtained at intervals from spore suspensions in 3 per cent HCl at different temperatures*

Time exposed	Water check	3% commercial hydrochloric acid		
		Flask 1	Flask 2	Flask 3
Room temperature 68–70° F.				
0 hours	2413	2360	2173	1813
12 "	2240	1347	1800	1493
24 "	2267	1387	933	1960
36 "	2173	823	423	1027
48 "	2120	627	507	1067
60 "	2560	680	1133	927
72 "	2253	720	440	867
96 "	2707	707	280	773
104 "	2187	413	253	587
90° F. temperature				
0 hours	2400	2360	1907	2560
8 "	2293	93	97	90
12½ "	2653	47	20	70
25 "	2347	0	10	0
36 "	2707	0	0	0
49½ "	2027	0	0	0
60 "	1067	0	0	0
100° F. temperature				
0 hours	4227	4480	4693	4467
1 "	5400	3040	4147	3760
3½ "	2333	0	13	0
7 "	4680	0	0	0
12 "	3353	0	0	0
24 "	1913	0	0	0
110° F. temperature				
0 hours	4253	3973	4133	4187
1 "	2827	1920	1307	573
2 "	3440	0	0	0
3 "	4493	0	0	0
5 "	3800	0	0	0
7 "	3503	0	0	0
12 "	0	0	0	0
120° F. temperature				
0 minutes	4600	4040	7600	8520
5 "	5060	160	20	1860
10 "	4500	0	0	20
15 "	1120	0	0	0
20 "	60	0	0	0
25 "	40	0	0	0
30 "	20	0	0	0
35 "	0	0	0	0

TABLE 3. *Number of viable spores of gray mold per cubic centimeter obtained at intervals from spore suspensions in soda ash cleaner, 75 lbs. per 100 gals., at different temperatures*

Time exposed	Water check	Sodium carbonate (soda ash)		
		Flask 1	Flask 2	Flask 3
Room temperature				
0 hours	5840	5660	5920	6660
12 "	8500	860	1580	1400
24 "	7480	20	880	680
48 "	6140	0	680	520
60 "	4880	0	420	360
72 "	6160	0	440	260
84 "	5260	0	160	80
101 "	6460	0	100	40
90° F. temperature				
0 hours	6160	8400	6820	6300
12 "	9200	4480	5900	4840
24 "	8300	1080	5980	4420
48 "	9860	80	1440	760
60 "	8500	80	600	280
72 "	7280	0	140	40
84 "	7040	0	20	20
101 "	6400	0	0	0
100° F. temperature				
0 hours	13960	12520	16160	14240
8 "	11680	14940	3100	16520
14 "	13200	2380	120	3400
24 "	10440	380	0	440
30 "	8880	160	0	120
36 "	6680	60	0	60
48 "	0	0	0	0
60 "	0	0	0	0
110° F. temperature				
0 "	11680	9440	8080	8960
2 "	4480	840	4260	2340
4 "	1340	100	1060	320
7 "	640	0	20	20
11 "	0	0	0	0
13 "	0	0	0	0
120° F. temperature				
0 minutes	4920	4400	4500	3640
10 "	3860	0	2560	3080
20 "	40	0	0	0
30 "	0	0	0	0
40 "	0	0	0	0

TABLE 4.—*Number of viable spores of gray mold per cubic centimeter obtained at intervals from spore suspensions in sodium silicate 75 lbs. per 100 gals., at different temperatures*

Time exposed	Water check	Sodium silicate	Time exposed	Water check	Sodium silicate
		Room temperature			
0 minutes	5400	5080	3 minutes	14800	4780
1 "	10420	9200	4 "	10900	1500
2 "	9750	7180	5 "	8000	360
		90° F. temperature			
0 minutes	9350	3600	3 minutes	9200	400
1 "	8100	3460	4 "	13400	320
2 "	8100	1700	5 "	20400	0
		100° F. temperature			
0 minutes	12900	3240	3 minutes	13900	0
1 "	15800	200	4 "	13000	0
2 "	12200	20	5 "	17700	0
		110° F. temperature			
0 minutes	7700	2140	1½ minutes	9900	20
½ "	6650	160	2 "	8650	0
1 "	4450	20	2½ "	9450	0
		120° F. temperature			
0 minutes	3750	180	1½ minutes	3750	0
½ "	7500	20	2 "	7650	0
1 "	7700	0	2½ "	7050	0

In plates made from the suspension of spores held in the water check for 2 hours at 110° F., table 2, colonies of limited size were formed in one plate in contrast to the normal spreading type (Fig. 2, A, B). The same type of colony formation appeared again in duplicate plates from one of the 3 per cent HCl flasks after 24 hours at room temperature (Fig. 2, C, D); but there were fewer colonies per plate and the colonies of limited size grew larger. The same phenomenon occurred again in the 3 per cent HCl series after 1 hour at 110° F. These 3 instances were the only ones observed in all the plates poured.

To determine whether the temperature factor was responsible for the abnormal colony formation, suspensions of spores from normal colonies were made and plates poured at different temperatures from 43° C.-73° C. at 5-degree intervals of temperature increases. The colony formation of limited size was not observed in these plates. Spore suspensions were made from these peculiar colonies and plated out, but the abnormal type could not be reproduced. From these tests this colony formation of limited size can be explained neither from the temperature factor nor on the basis of a

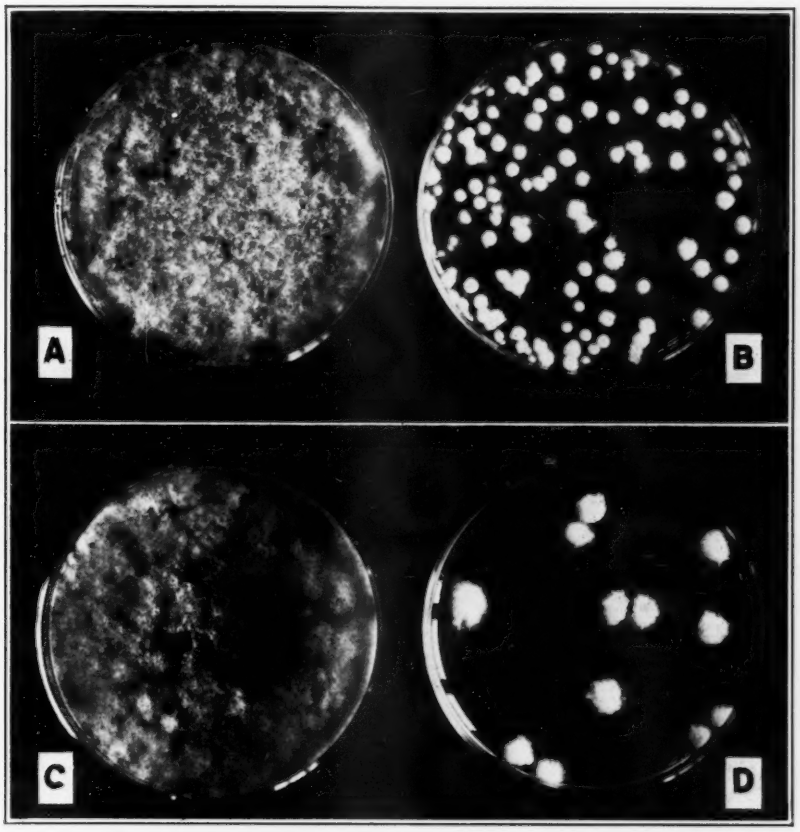


FIG. 2. Six-day-old colonies of *Botrytis cinerea* on 2 per cent potato-dextrose agar. A. Normal colonies. B. Abnormal colonies of limited size from water-check flask held 2 hours at 110° F. C. Normal colonies. D. Abnormal colonies of limited size from HCl flask held 24 hours at room temperature.

mutant. It appears to be due to some factor or condition entering into the methods used, the cause of which could not be determined.

DISCUSSION

Apple washing for the removal of spray residue by one of the chemical or wet processes has been practiced since 1927. Commercial hydrochloric acid, 1 per cent by weight or 3 per cent by volume, is most commonly used in apple washing. Various alkaline solvents have been tested, and those used most widely in recent years in this State are sodium carbonate and sodium silicate at concentrations of 75 to 100 pounds per 100 gallons of water. Recent washing experiments in Washington (9) and Oregon (11) have given good results with a double process wash, which consists of passing the fruit

through a washing unit containing sodium silicate and then through a hydrochloric acid wash, or the reverse. Robinson and Hatch (11) found hydrochloric acid was most effective at relatively low concentrations and at 100° to 110° F. They report sodium silicate a slower acting solvent than hydrochloric acid and recommend its use at higher concentrations, longer periods of time in the solvent, and at higher temperatures (110° to 120° F.). Overley *et al.* (9) report sodium silicate at a concentration of 80 to 100 pounds per 100 gallons of water at a temperature of 120° F. effective in the reduction of both lead and arsenic. A good foam was maintained in the solution and underneath brushes were used. Sodium silicate appeared to be more effective in removing the lead than soda ash. Baker and Heald (2) give data to show that sodium silicate reduced blue-mold decay to a greater extent than the other washing solutions tested. They do not recommend sodium silicate as an effective prevention of decay of apples if the inoculum is at a high level in the washing and rinsing tanks. In order to remove the residue the apples must remain in the solvent from 1 to 3 minutes, while the time required for rinsing ranges from 40 seconds to 1 minute. Temperatures of 100° F., 110° F., and 120° F. have been found most effective for the solvent bath.

Baker and Heald (1) have shown that both acid and alkaline cleaners, as used in commercial practice, killed off nearly all the blue-mold spores after an overnight stand. They also showed that the cleaners used were toxic to *Penicillium expansum* spores after shorter periods of exposure, and that the temperature alone, as well as the solvent, had a lethal effect on these spores.

In this investigation a study was made of the effect these cleaners have on gray-mold spores. The effect of both temperature and cleaners is summarized in table 5.

The cleaners were toxic on gray-mold spores at room temperatures and, as the solution were heated, the lethal effect on the spores increased with the rise in temperature.

The results (Table 5) indicate that the orchardist or fruit packer need have little concern about gray-mold contamination in his washing tank when using sodium silicate, at least when operating at the higher temperatures. This, however, would not protect against spores in the air or those present in the packing house. Chemically treated wraps have been found of commercial value in the prevention of botrytis rot of pears by Cooley and Crenshaw (4), while iodized wraps have shown promise in reducing some storage rots of apples (Tomkins, 13). This phase was not investigated for botrytis rot of apples.

When hydrochloric acid alone is used there might be a slight chance of contamination at the lower temperatures. The gray-mold spore load was

TABLE 5.—*Summary of the effect of cleaning solutions and of water at different temperatures on gray-mold spores*

Temperature	Per cent of spores killed after exposure stated							
	Per cent	Hrs.	Per cent	Hrs.	Per cent	Hrs.	Per cent	Min.
	Water Check		Hydrochloric acid (1 per cent by wt.)		Sodium carbonate 75 lbs. per 100 gals.		Sodium silicate 75 lbs. per 100 gals.	
Room	0.0	101	81.3	104	98.8	101	95.5	5
90° F.	0.0	101	99.9	25	99.9	84	52.7	2
			100.0	36	100.0	101	100.0	5
100° F.	52.2	36	99.9	3.5	99.9	36	98.7	1
	100.0	48	100.0	7.0	100.0	48	100.0	3
110° F.	94.6	7	69.1	1	99.9	7	97.6	0.5
	100.0	11	100.0	2	100.0	11	100.0	2
	Per cent	Min.	Per cent	Min.	Per cent	Min.	Per cent	Min.
120° F.	99.9	30	99.9	10	55.1	10	99.9	0.5
	100.0	35	100.0	15	100.0	20	100.0	1

greatly reduced by a heated solution and when followed by a running water rinse there should be very little gray-mold infection.

Sodium carbonate showed the least toxicity of any of the cleaners tested. At the common temperature of 110° F. there was considerable danger of contamination, if any large amount of gray-mold inoculum were present. Even at 120° F. the inoculum might accumulate from the spore load of the new apples or from an occasional decayed apple being introduced into the washing tank.

The introduction of tandem or double process washes and the widespread use of sodium silicate should greatly reduce mold contamination in the washing tanks. Washing with higher temperatures would have a tendency to reduce the spore load. A double wash at a temperature less than 100° F. with an alkali other than sodium silicate may offer some chance for gray-mold contamination. Observations[4] of commercial fruit in Yakima in 1934 indicate that temperatures above 120° F. injure the apples and open up avenues of entry for decay-producing fungi, since greater percentages of decay, mostly blue mold, were recorded from the apples washed at the higher temperatures ranging from 120° to 130° F. Gray mold, ranging from 0.2 to 1.4 per cent, occurred in 6 of the lots examined. This would indicate that some of the spores survived the action of the cleaning solutions at these

[4] Unpublished data collected by Paul Allen, former student in the Department of Plant Pathology.

temperatures. It also is possible that they were lodged in the lenticel basins, calyx, or stem cavities and did not come in direct contact with the cleaner.

SUMMARY

Washing tanks are one of the main sources of contamination of apples with gray mold, and it is desirable to find the most feasible sanitary practices to reduce this contamination.

The washing tank becomes contaminated with gray-mold spores from the surface of normal apples, from an occasional decayed apple that passes into the machine, and from the dust of the air.

The toxic action of hydrochloric acid, sodium carbonate, sodium silicate, and water on gray-mold spores was determined. Temperatures of the solutions ranged from room to 120° F.

Spores of *Botrytis cinerea* were held in suspension in flasks and subjected to both chemical toxicity and temperature fluctuations. Spore viability was ascertained by the ability to form colonies on 2 per cent dextrose-potato agar.

Abnormal gray-mold colonies of limited size developed in contrast to the normal spreading colonies when duplicate plates were made from the same flask. The cause of this phenomenon was not determined.

Cleaning solutions used in the removal of spray residue have a toxic effect on gray-mold spores. Of the 3 cleaners commonly used in the removal of spray residue, sodium silicate was the most toxic to gray mold, hydrochloric acid less toxic, and sodium carbonate the least so. Toxicity increased with a prolongation of exposure to the chemical and a rise in temperature.

The temperatures at which the cleaning solutions were used had a marked toxic action on gray-mold spores. As the solutions were heated from 90° F. to 120° F., the percentage of spores killed increased.

The results indicate that the orchardist or fruit packer need have little concern about gray-mold contamination during the washing operations when using sodium silicate, at least when operating at the higher temperatures.

The introduction of tandem or double process washes at the higher temperatures and the widespread use of sodium silicate greatly reduces the chances of gray-mold contamination in the washing tanks.

DEPARTMENT OF PLANT PATHOLOGY,
STATE COLLEGE OF WASHINGTON,
PULLMAN, WASHINGTON.

LITERATURE CITED

1. BAKER, K. F., and F. D. HEALD. Some problems concerning blue mold in relation to cleaning and packing of apples. Phytopath. 22: 879–898. 1932.

2. BAKER, K. F., and F. D. HEALD. Investigations on methods of control of the blue-mold decay of apples. Washington Agr. Expt. Sta. Bull. 304. 1934.
3. BRIEN, R. M. The fungi causing rots of stored apples in New Zealand. New Zeal. Jour. Agr. **48**: 143–149. 1934.
4. COOLEY, J. S., and J. H. CRENSHAW. Control of Botrytis rot of pears with chemically treated wraps. U. S. Dept. Agr. Circ. 177. 1931.
5. DIEHL, H C., D. F. FISHER, H. HARTMAN, J. R. MAGNESS, and R. H. ROBINSON. Removal of spray residue from apples and pears in the Pacific Northwest. U. S. Dept. Agr. Circ. 59. 1929.
6. FISHER, D. F., and E. L. REEVES. Arsenical and other fruit injuries of apples resulting from washing operations. U. S. Dept. Agr. Tech. Bull. 245. 1931.
7. HEALD, F. D., J. R. NELLER, F. L OVERLY, G. D. RUEHLE, and W. A. LUCE. Arsenical spray residue and its removal from apples and pears. Washington Agr. Expt. Sta. Bull. 226. 1928.
8. ————————, and G. D. RUEHLE. The rots of Washington apples in cold storage. Washington Agr. Expt. Sta. Bull. 253. 1931.
9. OVERLEY, F. L., J. L. ST. JOHN, E. L. OVERHOLSER, and K. GROVES. Lead and arsenic spray residue removal from apples. Washington Agr. Expt. Sta. Bull. 286. 1933.
10. PENTZER, W. T. Removal of lead and arsenic spray residues from New York apples. New York (Cornell) Agr. Expt. Sta. Bull. 604. 1934.
11. ROBINSON, R. H., and M. B. HATCH. The removal of lead and arsenic spray residues from apples and pears. Oregon Agr. Expt. Sta. Bull. 317. 1933.
12. SHEAR, E. V. Washing fruit to remove spray residue in the Hudson Valley. New York State Agr. Expt. Sta. Bull. 575. 1929.
13. TOMKINS, R. G. Iodized wraps for the prevention of rotting of fruit. Jour. Pomol. and Hort. Sci. **12**: 311–320. 1934.

STUDIES ON THE EFFECT OF CARBORUNDUM AS AN ABRASIVE IN PLANT VIRUS INOCULATIONS[1]

T. E. Rawlins and C. M. Tompkins[2]

(Accepted for publication June 15, 1935)

INTRODUCTION

Some viruses, such as the tobacco mosaics and cucumber mosaic viruses may be readily transmitted by rubbing infective juice on the leaves of susceptible hosts, as recommended by Holmes (10), but many have been regularly transmitted only by insects or grafts. One of the most important viruses, difficult to transmit regularly by the use of Holmes' method, is the spotted-wilt virus, which infects a number of hosts but is particularly destructive to tomato and lettuce. Other viruses that have not been transmitted regularly by the rubbing method are the sugar-beet, crucifer, broad-bean, and celery-mosaic viruses, all of which occur in California.

Failure to regularly transmit these viruses may be due to failure of the inoculation methods to produce minute openings in the host cell walls. It is supposed that very small openings in the host cells admit virus particles but do not injure the cells to such an extent as to render them unfavorable for virus multiplication. Evidence in favor of this hypothesis has been furnished by workers who have used sand as an abrasive. Fajardo (6) found that sand rubbed on the leaves with the bean-mosaic virus increased infection; and Fajardo and Maranon (7) used this method successfully in transmitting mosaic of Sincamas, *Pachyrrhizus erosus* L. Urban. Samuel and Bald (18) found that 120-mesh quartz river sand added to a suspension of the yellow tobacco-mosaic virus greatly increased the number of primary lesions produced on various species of Nicotiana.

It, therefore, appeared reasonable to assume that certain other abrasives composed of smaller and sharper crystals might be more satisfactory than sand in producing the supposedly necessary minute openings in inoculated cells. It was found that powdered carborundum crystals possessed the desired structure. A note on the use of carborundum has already been published (16). Further experiments carried out to test this hypothesis are described in detail.

MATERIALS AND METHODS

Young, healthy plants, grown from seed in the greenhouse and later transplanted to 6-inch porous clay pots, were used in all inoculation experi-

[1] Contribution from the Division of Plant Pathology, University of California, Berkeley, California.

[2] The writers are indebted to Professor Ralph E. Smith and Dr. Max W. Gardner for helpful suggestions and advice.

ments. The greenhouse temperature varied from 55° to 65° F. Weekly fumigation of the greenhouse was given by heating a small quantity of nicotine sulphate in a shallow pan. This gave good control of aphids and thrips.

In preliminary trials various abrasives, such as finely ground but ungraded quartz sand, powdered silicic acid and jewelers' rouge, were tested. Since the results obtained by the use of these substances did not seem promising, further tests were limited to a 600–mesh, powdered carborundum. This substance was dusted over the upper surface of the leaves to be inoculated by means of a salt shaker provided with small openings. No attempt was made to distribute the abrasive uniformly when applying, since more or less uniform distribution resulted later when the virus suspension was wiped on the leaf.

Virus suspensions were prepared by grinding leaves from young plants showing the first symptoms of disease in a mortar lined with a square of cheesecloth. After grinding to a pulp the juice was pressed through the cheesecloth. Except where noted later, the extracted, undiluted juice was applied without centrifuging or purifying.

A sterile, 8–inch pot label was held under the leaf to be inoculated. A small ball of sterilized absorbent cotton, held in forceps, was dipped into the virus suspension and then twice drawn lightly over the leaf surface in one direction, usually from the base to the tip of the leaf. This operation tended to distribute the particles of carborundum and virus more or less uniformly over the leaf surface, but did not cause any macroscopic injury to the leaf tissues. Conspicuous injury does occur if the carborundum is rubbed into the leaf by heavy pressure on the cotton. All the leaves on each test plant, usually 2 or 3 in number, were inoculated.

Experimental

In studying the efficacy of carborundum for rendering plants susceptible to virus infection, a number of diseases were investigated in order to determine how generally the method may be applied. Certain experimental details and the results are given in table 1.

Spotted Wilt

This virus was transferred from lettuce to lettuce and from lettuce to tomato. As is shown in table 1, the use of carborundum during inoculation gave a very significant increase in infection of both hosts.

The spotted-wilt virus produces primary local lesions around the leaf cells where infection has occurred in the case of *Nicotiana glutinosa*. Leaves of this plant were inoculated in the usual way, with and without carborundum. As is shown in table 2, although enormous variation is observed

TABLE 1.—*Transmission of different viruses with and without the use of carborundum*

Virus used	Source of inoculum	Host inoculated	Date inoculated	Date read	Mode of inoculation			
					Carborundum		No carborundum	
					Number of plants inoculated	Number of plants infected	Number of plants inoculated	Number of plants infected
Spotted-wilt	Diseased Romaine lettuce plant, naturally infected. San Pablo, California	Lettuce	4–18–34	5– 4–34	20	8	20	0
			5– 1–34	5–21–34	20	18	20	4
			12–31–34	1–20–35	20	14	20	0
Spotted-wilt	"	Tomato	12–24–34	1–20–35	20	16	20	6
Broad-bean mosaic	Diseased broad-bean plant, naturally infected. Niles, California	Broad bean	5–31–34	6–16–34	44	31	39	1
			7–19–34[a]	8– 6–34	27	19	32	7
Celery-mosaic	Diseased celery plant, artificially infected with a celery-mosaic virus from Southern California	Celery	10– 8–34	10–23–34	25	23	25	3
			10–31–34	11–15–34	24	15	23	3
Cauliflower-mosaic	Diseased cauliflower plant, naturally infected. Alvarado, California	Cauliflower	6– 5–34	6–20–34	20	15	20	0
			12–20–34	1– 8–35	25	23	25	0
Sugar-beet mosaic	Diseased sugar beet, naturally infected. Berkeley, California	Sugar beet	8–23–34	9– 7–34	70	50	70	17
			10–16–34	10–29–34	50	37	50	14
California-aster or celery-yellows	Diseased aster plant, experimentally infected. Berkeley, California	Aster	10– 8–34	11–20–34	25	0	25	0
			11– 8–34	12–10–34	20	0	20	0

[a] 13 noninoculated broad-bean plants, used as controls, remained healthy.

TABLE 1.—*Continued*

Virus used	Source of inoculum	Host inoculated	Date inoculated	Date read	Mode of inoculation			
					Carborundum		No carborundum	
					Number of plants inoculated	Number of plants infected	Number of plants inoculated	Number of plants infected
Lettuce brown-blight[b]	Diseased lettuce plants, naturally infected. Salinas, California	Lettuce	10–22–34	11–22–34	20	0	20	0
			11– 1–34	12– 1–34	20	0	20	0
Lettuce big-vein[b]	Diseased lettuce plants, naturally infected. San Pablo, California	Lettuce	3–17–34	4–20–34	15	0	15	0
			4–25–34	6– 2–34	20	0	20	0
Sugar-beet curly-top	Diseased sugar-beet leaves from naturally infected plant. San Ardo, California	Sugar beet	6– 9–34	7–16–34	25	0	25	0
	Diseased sugar-beet root from naturally infected plant. San Ardo, California		6–30–34	8– 7–34	25	0	25	0
	Diseased sugar-beet root from naturally infected plant. San Ardo, California		8–14–34	9–21–34	25	0	50	0

[b] Not yet definitely identified as virus diseases.

in the results, the carborundum caused a greatly increased total number of local lesions.

TABLE 2.—*Inoculations of Nicotiana glutinosa,*[a] *with and without carborundum, with the spotted wilt virus*

With carborundum		No carborundum	
Plant no.	No. local lesions	Plant no.	No. local lesions
1	2	17	0
2	4	18	0
3	21	19	0
4	12	20	8
5	12	21	1
6	4	22	5
7	7	23	3
8	58	24	12
9	7	25	3
10	6	26	1
11	6	27	1
12	106[b]	28	4
13	10	29	2
14	7	30	2
15	13	31	5
16	165	32	1
Totals	440†		48††

[a] Inoculated 12–5–34. Read 12–13–34.

[b] Systemic infection. Plant died on the 8th day.

† Average number local lesions per plant when carborundum was used = 27½.

†† Average number local lesions per plant when carborundum was not used = 3.

Broad-bean Mosaic

Studies of the mosaic of broad-bean in Germany by Böning (3) and in Japan by Fukushi (8) and Imai (11) have shown the difficulties encountered in trying to obtain infection by mechanical means. Böning did not succeed in inducing the disease by artificial means, while Imai secured only 34 per cent infection.

As shown in table 1, a high percentage of infection resulted only when carborundum was used, demonstrating the efficacy of this material as an aid in transmitting broad-bean mosaic.

A California Celery Mosaic

The celery-mosaic virus with which we worked was obtained from diseased celery plants collected at Venice, California. This virus was recently described by Severin and Freitag (20). As is shown in table 1, this disease is

difficult to transmit reguarly by Holmes's method. The carborundum method increased the percentage of transmission approximately 5 to 7 times.

Cauliflower Mosaic

This recently described virosis, (22) appears to be different from any of the crucifer viruses previously reported in the literature. As is shown in table 1 it was not transmitted by Holmes's method but was transmitted to 38 of the 45 inoculated plants by means of the carborundum method. Transmission has never been obtained by rubbing without the carborundum.

Sugar-beet Mosaic

Robbins (17) and Böning (2) failed to infect healthy sugar beets with the virus of sugar-beet mosaic by juice inoculations. Jones (14) tried unsuccessfully to transmit the mosaic disease of garden beet by mechanical means. Verplancke (23) and Smith (21) secured transmission by rubbing, while Hoggan (9) reported systemic infection as a result of severe rubbing.

Our preliminary test with the rubbing method also gave negative results. In the first test with carborundum, 71 per cent of the plants were infected as compared with 24 per cent where the abrasive was omitted. In the second test, 74 per cent infection with carborundum, as against 28 per cent without, was obtained. Thirty healthy noninoculated sugar-beet seedlings served as controls in each series to test the possibility of accidental infection. All control plants remained healthy. It is, therefore, evident that this virus may be readily transmitted by the use of carborundum.

Aster Yellows[3]

All workers who have attempted to transmit the two strains of aster yellows by juice inoculations have been unsuccessful. As is evident from the results shown in table 1, the carborundum method also was unsatisfactory in transmitting the California-aster or celery-yellows virus. It is possible that this virus, like the curly-top virus, is confined to the phloem and that any successful method of inoculation must involve the injection of the virus into the phloem.

An attempt was made to introduce the virus into the phloem by cutting away the lower portion of the midrib and larger veins with a sharp razor blade to expose the phloem and then rubbing the cut tissues with 600-mesh carborundum and virus suspension by means of a glass spatula. Five aster plants were thus inoculated but no infection resulted in any of the plants.

[3] Grateful acknowledgment is made to Dr. H. H. P. Severin and Dr. Julius H. Freitag for having supplied the curly-top and aster-yellows viruses, respectively.

Curly Top[4]

Numerous attempts have been made to develop a satisfactory method of mechanical inoculation for the transmission of the curly top virus. Tests were made by Carsner and Stahl (4), Severin (19), and Dana (5), but only a very small percentage of infection resulted, so that the principal means of transmission continues to be that of the insect vector.

After inoculation with the curly-top virus by the carborundum method, sugar-beet seedlings in each of 3 series were held for 5 weeks; none of the plants became infected. The negative results were not wholly unexpected, since Bennett (1) has shown that the path of the virus is in the phloem. It is believed that no carborundum particles reached the phloem, which may account for lack of infection.

As in the case of the California aster or celery yellows, an attempt was made to introduce the virus into the phloem of beet leaves by cutting away the lower portion of the midrib and larger lateral veins to expose the phloem and then rubbing virus and 600-mesh carborundum over the cut tissues by means of a glass spatula. Fifteen beet plants were inoculated by this method but none of the plants became infected.

Brown Blight of Lettuce

Jagger (12) found brown blight of lettuce to be soil-borne but he was unable to transmit it by other means. Since the symptoms of this disease suggest that it may be a virosis an attempt was made to transmit it by means of the carborundum method. As is shown in table 1, infection did not result from any of the inoculations.

Big-vein Disease of Lettuce

Jagger and Chandler (13) have recently reported that big vein of lettuce also is soil-borne. Since this disease also exhibits symptoms occurring in some viroses an attempt was made to transmit the disease to healthy lettuce plants by means of the carborundum method. As is evident from the results shown in table 1, all of the inoculations were unsuccessful.

Histological Studies

An attempt was made to gain evidence regarding the action of the carborundum particles on the host cells by cutting sections of cauliflower leaves that had been inoculated with cauliflower mosaic virus after the leaves had been dusted with carborundum powder. The inoculated tissues were fixed at times varying from 1 to 29 hours after inoculation; Karpechenko's solution and Flemming's strong solution diluted with equal parts of water were

[4] See footnote 3.

found to be the most satisfactory fixing solutions. After fixation the tissues were dehydrated with glycerin and infiltrated in turn with butyl alcohol and paraffin, as previously reported (15). When sectioning, the block was always so oriented that the microtome knife would cut through the leaf before striking any of the crystals on the upper surface of the leaf. This was done to insure that no crystals should be carried into the leaf cells by the knife. The sections were cut rather thick (15 μ to 20 μ) in order that minimum disturbance of the carborundum crystals by the knife should result. The sections were stained with Heidenhain's iron alum-hematoxylin combination. In certain cells the polarizing microscope was found to aid in observing the penetration of the cell walls by the crystals.

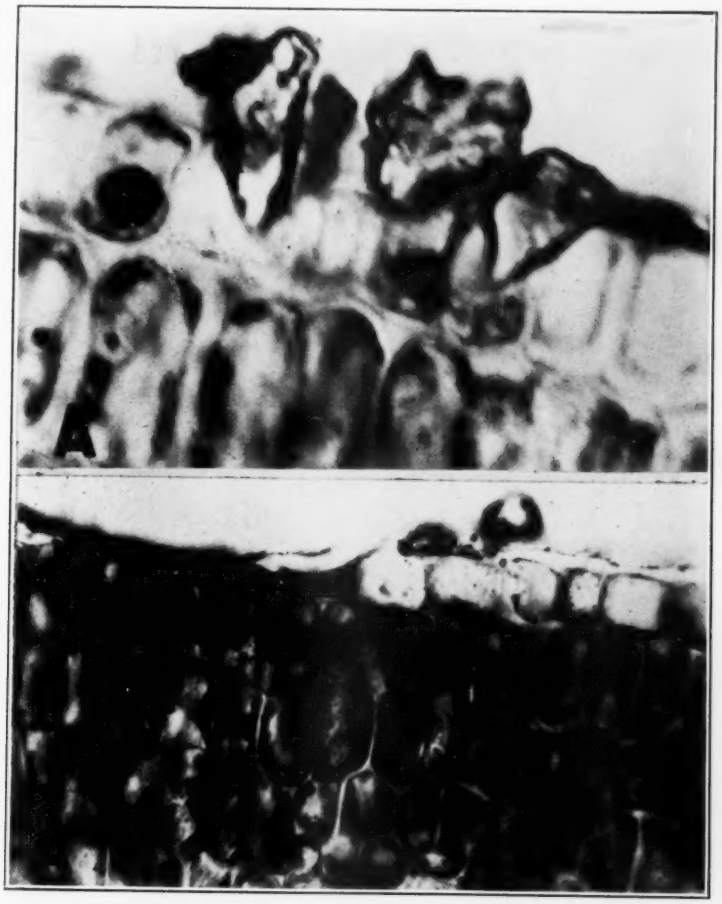


FIG. 1. Sections of cauliflower leaves on which carborundum was used during inoculation. A. Crystals that have pierced the epidermal cells. B. Collapsed epidermal cells, killed by the carborundum.

As is seen in figure 1, A, numerous epidermal cells that apparently had been penetrated by the carborundum crystals were observed. It is also evident that the points of the crystals are often small relative to the size of the cells and should therefore be able to penetrate the cells with a minimum of injury. In certain cases, as shown in figure 1, B, some of the epidermal cells were killed by the carborundum and have collapsed.

Since the histological work has shown that epidermal cells are frequently pierced by the sharp points of the crystals, it is reasonable to suppose that during the inoculation procedure some of the carborundum crystals produce small openings that allow virus particles to enter but do not injure the cells sufficiently to prevent virus multiplication.

SUMMARY

By the use of powdered carborundum during inoculation, it was possible to transmit readily a number of viruses that have been found difficult to transmit by other methods. Among these viruses are those causing spotted wilt of tomato and lettuce, broad-bean mosaic, a California celery mosaic, cauliflower mosaic and sugar-beet mosaic. Attempts to transmit curly top of sugar beet, California aster or celery yellows, and brown blight and big vein of lettuce were unsuccessful.

The 600-mesh grade of carborundum apparently was effective.

Histological studies demonstrated that in leaves inoculated by the carborundum method the epidermal cells frequently are pierced by the carborundum crystals and that the points of the crystals are small relative to the size of the cells. This evidence favors the hypothesis that the carborundum crystals may pierce leaf cells and allow virus particles to enter but in certain cases do not injure the pierced cells sufficiently to prevent virus multiplication in these cells.

UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA

LITERATURE CITED

1. BENNETT, C. W. Plant-tissue relations of the sugar-beet curly-top virus. Jour. Agr. Research [U. S.] **48**: 665–701. 1934.
2. BÖNING, K. Die Mosaikkrankheit der Rübe. Forsch. auf dem Geb. Pflanzenkrank. u. Immunität Pflanzenr. **3**: 81–128. 1927.
3. ————. Die Mosaikkrankheit der Ackerbohne (*Vicia faba* L.). Ein Beitrag zu dem Mosaik der Papilionaceen. Forsch. auf dem Geb. Pflanzenkrank. u. Immunität. Pflanzenr. **4**: 43–111. 1927.
4. CARSNER, E., and C. F. STAHL. Progress report on curly-top of the sugar beet. (Abstract) Phytopath. **14**: 122–123. 1924.
5. DANA, B. F. Some experiments with mechanical transmission of the curly-top virus. (Abstract) Phytopath. **22**: 997–998. 1932.

6. FAJARDO, T. G. Studies on the mosaic disease of the bean (*Phascolus vulgaris* L.). Phytopath. **20**: 469–494. 1930.
7. ————, and J. MARAÑON. The mosaic disease of Sincamas, *Pachyrrhizus erosus* (Linnaeus) Urban. Philippine Jour. Sci. **48**: 129–142. 1932.
8. FUKUSHI, T. [On the mosaic disease of broad beans.] Byôchü-Gai Zasshi (Jour. Plant Protect.) [Tokyo] **17**: 707–712. 1930. [In Japanese. Eng. summary in Japan. Jour. Bot. **5**: (90). 1931.]
9. HOGGAN, I. A. Some viruses affecting spinach, and certain aspects of insect transmission. Phytopath. **23**: 446–474. 1933.
10. HOLMES, F. O. Inoculating methods in tobacco mosaic studies. Bot. Gaz. **87**: 56–63. 1929.
11. IMAI, Y. Studies on the transmission of broad bean mosaic. Sapporo Nat. Hist. Soc. Trans. **13**: 241–245. 1934.
12. JAGGER, I. C. Lettuce breeding for disease resistance progresses rapidly. Brown blight. U. S. Dept. Agr. Yearbook, **1931**: 348–350. 1931.
13. ————, and N. CHANDLER. Big vein, a disease of lettuce. Phytopath. **24**: 1253–1256. 1934.
14. JONES, L. K. The mosaic disease of beets. Washington Agr. Expt. Sta. Bull. 250. 1931.
15. RAWLINS, T. E. Phytopathological and botanical research methods. 156 p. J. Wiley & Sons. New York. 1933.
16. ————, and C. M. TOMPKINS. The use of carborundum as an abrasive in plant-virus inoculations. (Abstract) Phytopath. **24**: 1147. 1934.
17. ROBBINS, W. W. Mosaic disease of sugar beets. Phytopath. **11**: 349–365. 1921.
18. SAMUEL, G., and J. G. BALD. On the use of the primary lesions in quantitative work with two plant viruses. Ann. Appl. Biol. **20**: 70–99. 1933.
19. SEVERIN, H. H. P. Curly leaf transmission experiments. Phytopath. **14**: 80–93. 1924.
20. ————, and J. H. FREITAG. California celery mosaic diseases. (Abstract) Phytopath. **25**: 891. 1935.
21. SMITH, KENNETH M. The mosaic disease of sugar-beet and related plants. Jour. Min. Agr. [Gt. Brit.] **41**: 269–274. 1934.
22. TOMPKINS, C. M. A destructive virus disease of cauliflower and other crucifers. (Abstract) Phytopath. **24**: 1136–1137. 1934.
23. VERPLANCKE, G. Les maladies à virus filtrants de la betterave. Suer. Belge **49**: 121–127. 1929.

THE INFLUENCE OF SEED HULLING ON LOOSE SMUT IN NATURALLY INOCULATED OATS

V. F. Tapke

(Accepted for publication July 8, 1935)

INTRODUCTION

In studies on the loose smut of oats (*Ustilago avenae* (Pers.) Jensen) the methods of artificial seed inoculation commonly employed in the past have consisted chiefly in applying dry spores to the surface of nonhulled or hulled[1] seed as noted in the review by Stanton *et al.* (9). In 1922, however, Zade (10) first showed that, under natural conditions of inoculation, the effective inoculum is chiefly mycelium that invades the inner parenchymatous layers of the hulls. This mycelium results from the germination of spores carried within the flowers during anthesis. The principal facts of Zade's discovery have since been repeatedly confirmed (1, 2, 3, 7, 8, 11). A striking difference, therefore, exists between the artificial and natural methods of inoculation with respect to the relative positions of inoculum and hulls. In the artificial methods, the inoculum comes into direct contact with the soil when the seed is sown. The hulls can play no part in shielding it against the full force of conditions inimical to infection. In the natural method of inoculation, however, the inoculum is protected by the enclosing hulls. Considering their siliceous nature and tight appression to the caryopsis, it would seem that they might function importantly in the case of naturally inoculated oats in protecting the inoculum against desiccation, excess moisture, sudden changes in temperature, and other unfavorable soil factors, during the critical period of seed germination and seedling infection. The influence of the hulls in this respect apparently has not been determined; obviously, a factor that might make an important difference in the results obtained from artificial and natural methods of seed inoculation. A study of the problem, therefore, seemed desirable. The procedure in the following experiments involved the hull removal of naturally inoculated seed. Further opportunity, therefore, was afforded to determine whether the effective inoculum resided chiefly in the inner parenchymatous lining or other parts of the hulls or in the pericarp of the caryopsis. As noted later, previous investigators are not in accord on this point. The results of the writer's investigation are presented in the following pages.

RELATED INVESTIGATIONS

In the loose smut of oats, the diseased panicles emerge and shed their smut spores during the emergence and anthesis of the normal smut-free

[1] In the present report, the term *hulled* signifies that the hulls have been removed from the mature seed by hand. The term *nonhulled* refers to oat seed with the hulls intact.

heads. Floral inoculation of the host commonly occurs throughout this period consequent upon the opening of the glumes and exposure of the flowers to errant smut spores.

Previous to Zade's discovery in 1922 (10), the importance of the positional relation of seed inoculum to smut infection in oats was not realized. Since 1922, however, Zade (11), Arland (1), Diehl (2), Roesch (7), Gage (3), and Sampson (8) have further contributed, showing that spores on the outside of the hulls play a minor part in causing loose smut in oats under natural conditions of inoculation. Their research also has shown that the spores entrapped within the glumes do not remain dormant, as formerly believed, but normally begin to germinate within a few hours and eventually give rise to a mycelium that invades various structures of the seed. Zade and Arland (13) concluded that under their ecological conditions, in Leipzig, Germany, chiefly the parenchyma of the hulls is invaded by the mycelium.

Gage (3) found that removal of the hulls of seed from hand-inoculated flowers effected only a small reduction in the percentage of smut. He concluded, therefore, that the effective inoculum resulting from floral inoculation consists in mycelium that eventually invades chiefly the pericarp of the caryopsis. Johnston (6), however, obtained high reductions in the amount of smut in 5 naturally inoculated lots as a result of seed hulling. He concluded that this was due to the fact that most of the inoculum was carried on or in the hulls and, therefore, was removed with them in the process of hulling. It is interesting to note that Gage's results were obtained under greenhouse conditions very favorable to smut infection and that Johnston's results were obtained under relatively unfavorable field conditions. It, therefore, seemed possible that environmental conditions might be related to the influence of seed hulling on loose smut in naturally inoculated oats. It was evident also that if it were found that the hulls have an important influence on the enclosed inoculum of florally inoculated seed, this knowledge would aid in evaluating the reliability of results from various artificial methods of seed inoculation in which the inoculum is not enclosed by the hulls. As noted later, some apparently valid objections to the methods of applying dry spores to nonhulled or to hulled seed previously have been raised on other bases.

MATERIALS AND METHODS

From a collection of naturally inoculated seed lots from the crop of 1928, 10 lots were selected that were infested largely or entirely with the loose smut, as shown in a greenhouse planting in the winter of 1928–29. The 10 lots were collected from 5 States and represented 5 different varieties, strains or mixtures. Sowings were made in the field and greenhouse at the

Arlington Experiment Farm, Rosslyn, Virginia, and in an irrigated field at Aberdeen, Idaho.

In all of the tests the hulls were removed within a period of 3 weeks or less before sowing. In the field experiment at the Arlington farm, 250 nonhulled and 250 hulled seeds of each of the 10 lots were sown in adjacent rod rows 1 ft. apart. The field planting at Aberdeen was similar except that only 7 lots were used and the rows were 7 feet long, 100 seeds to the row. In the greenhouse experiment at the Arlington farm, 100 nonhulled and 100 hulled seeds of each of the 10 lots were sown in an alternating series of 8 42-inch rows, 25 seeds per row.

RESULTS

The results of the experiment are presented in table 1.

Table 1 shows that seed hulling consistently produced some reduction in the amount of smut. However, the degree of reduction varied widely, both within individual lots grown under different conditions and between different lots grown under similar conditions. The data, therefore, have little value in determining whether the inoculum in the hulls or pericarp is accountable for most of the smut in oats caused by *Ustilago avenae*.

Within individual lots grown under the different field and greenhouse conditions, the differences in smut reduction due to seed hulling were particularly striking, ranging from as little as 9.4 per cent in the case of lot 1 to as much as 88.1 per cent in lot 3. In the latter the percentage reductions in smutted plants resulting from hulling were 2.1 in the greenhouse at Rosslyn, 24.3 in the field at Aberdeen, and 90.2 in the field at Rosslyn. The greenhouse data indicate that the effective inoculum resided largely in the pericarp of the caryopsis, as removal of the hulls produced only a slight reduction in the percentage of smutted plants. The Rosslyn field data, however, indicate that the effective inoculum resided largely in the hulls. Similar inconsistencies are obtained in comparing the different lots grown under similar conditions. Hulling effected reductions in the percentage of smutted plants ranging from 24.3 to 73.2 in the field at Aberdeen, from 72 to 100 in the field at Rosslyn, and from 2.1 to 91.1 in the greenhouse at Rosslyn.

Further examination of table 1 shows that, in general, relatively unfavorable conditions for smuttedness were more acutely reflected in plants from hulled seed than in plants from nonhulled seed. In the ten lots grown at Rosslyn, the average percentages of smutted plants from nonhulled seed were 13.8 in the field and 43.6 in the greenhouse. Under the relatively unfavorable field conditions, the reduction in smutted plants due to seed hulling was 89.9 per cent. In the greenhouse the reduction was only 48.2 per cent. In the 7 lots grown in the field, both at Aberdeen and Rosslyn, the average, 14.8 per cent, of smutted plants from nonhulled seed at Rosslyn

TABLE 1.—*Influence of hulling the caryopsis on loose smut in oats. Plants grown from naturally inoculated seed sown in the field at Aberdeen, Idaho, and at Rosslyn, Virginia (Arlington Experiment Farm), in the spring of 1929 and in the greenhouse at Rosslyn in the winter of 1929–30. Seven lots were sown at Aberdeen and 10 lots in the field and greenhouse at Rosslyn*

Seedlot number, variety, and source	Location of experiment	Seed nonhulled (n) or hulled (h)	Plants matured			Reduction in smutted plants due to seed hulling
			Total	Smutted		
			Number	*Number*	*Per cent*	*Per cent*
1 Green Russian from Iowa	Field, Rosslyn, Va.	(n)	197	13	6.6	90.9
		(h)	163	1	0.6	
	Greenhouse, Rosslyn, Va.	(n)	97	12	12.4	81.5
		(h)	87	2	2.3	
2 Green Russian from Iowa	Field, Aberdeen, Ida.	(n)	73	9	12.3	30.1
		(h)	81	7	8.6	
	Field, Rosslyn, Va.	(n)	215	25	11.6	85.3
		(h)	181	3	1.7	
	Greenhouse, Rosslyn, Va.	(n)	99	48	48.5	21.0
		(h)	94	36	38.3	
3 Green Russian from Iowa	Field, Aberdeen, Ida.	(n)	78	9	11.5	24.3
		(h)	69	6	8.7	
	Field, Rosslyn, Va.	(n)	195	26	13.3	90.2
		(h)	158	2	1.3	
	Greenhouse, Rosslyn, Va.	(n)	98	42	42.9	2.1
		(h)	88	37	42.0	
4 Frazier from Texas	Field, Aberdeen, Ida.	(n)	85	13	15.3	73.2
		(h)	73	3	4.1	
	Field, Rosslyn, Va.	(n)	231	27	11.7	100.0
		(h)	205	0	0	
	Greenhouse, Rosslyn, Va.	(n)	89	41	46.1	91.1
		(h)	73	3	4.1	
5 A mixture from New Jersey	Field, Aberdeen, Ida.	(n)	82	19	23.2	57.3
		(h)	71	7	9.9	
	Field, Rossyln, Va.	(n)	202	49	24.3	72.0
		(h)	207	14	6.8	
	Greenhouse, Rosslyn, Va.	(n)	94	61	64.9	52.9
		(h)	85	26	30.6	
6 Kanota from Kansas	Field, Aberdeen, Ida.	(n)	92	8	8.7	27.6
		(h)	64	4	6.3	
	Field, Rosslyn, Va.	(n)	247	27	10.9	96.3
		(h)	223	1	0.4	
	Greenhouse, Rosslyn, Va.	(n)	100	21	21.0	47.6
		(h)	100	11	11.0	
7 Kanota from Kansas	Field, Aberdeen, Ida.	(n)	88	18	20.5	48.8
		(h)	76	8	10.5	
	Field, Rosslyn, Va.	(n)	242	43	17.8	100.0
		(h)	237	0	0	
	Greenhouse, Rosslyn, Va.	(n)	75	39	52.0	72.1
		(h)	62	9	14.5	

TABLE 1.—(*Continued*)

Seedlot number, variety, and source	Location of experiment	Seed nonhulled (n) or hulled (h)	Plants matured			Reduction in smutted plants due to seed hulling
			Total	Smutted		
			Number	*Number*	*Per cent*	*Per cent*
8 Kanota from Kansas	Field, Rosslyn, Va.	(n)	225	16	7.1	93.0
		(h)	211	1	0.5	
	Greenhouse, Rosslyn, Va.	(n)	98	24	24.5	74.3
		(h)	80	5	6.3	
9 Swedish Select from Wisconsin	Field, Aberdeen, Ida.	(n)	61	9	14.8	34.4
		(h)	72	7	9.7	
	Field, Rosslyn, Va.	(n)	220	32	14.5	78.6
		(h)	225	7	3.1	
	Greenhouse, Rosslyn, Va.	(n)	84	74	88.1	33.3
		(h)	97	57	58.8	
10 Kanota from Kansas	Field, Rosslyn, Va.	(n)	226	46	20.4	100.0
		(h)	209	0	0	
	Greenhouse, Rosslyn, Va.	(n)	90	41	45.6	86.2
		(h)	79	5	6.3	
Lots 2, 3, 4, 5, 6, 7, and 9	Field, Aberdeen, Ida.	(n)	559	85	15.2	45.4
		(h)	506	42	8.3	
	Field, Rosslyn, Va.	(n)	1552	229	14.8	87.2
		(h)	1436	27	1.9	
	Greenhouse, Rosslyn, Va.	(n)	639	326	51.0	41.4
		(h)	599	179	29.9	
All lots	Field, Rosslyn, Va.	(n)	2200	304	13.8	89.9
		(h)	2019	29	1.4	
	Greenhouse, Rosslyn, Va.	(n)	924	403	43.6	48.2
		(h)	845	191	22.6	

was only slightly less than the 15.2 per cent at Aberdeen. The average reduction in smut resulting from seed hulling at Rosslyn (87.2 per cent) was, however, almost twice that at Aberdeen (45.4 per cent). Finally, comparison of data from the 7 lots grown in the field at Aberdeen and the greenhouse at Rosslyn shows similar results, but in much less striking degree. The average percentages of smut from nonhulled seed were 15.2 at Aberdeen and 51.0 at Rosslyn. The average reductions in smut resulting from seed hulling were 45.4 per cent at Aberdeen and 41.4 per cent at Rosslyn. It is interesting also to note that the divergent results of Gage (3) and Johnston (6) referred to above may be explained on the basis that conditions unfavorable to smut are more pronounced in plants from hulled seed than in those from nonhulled seed. Gage obtained a reduction in smutted plants of only 8.2 per cent from hulled seed under greenhouse conditions that resulted in 67.0 per cent of smutted plants from the nonhulled seed. Johnston, however, obtained reductions up to 100 per cent from seed hulling, under field condi-

tions relatively unfavorable to the development of smut in plants from the nonhulled seed.

The data of table 1 show further that hulled seed of all of the lots produced some smutted plants; evidence that at least a portion of the inoculum resided in the caryopses of these lots.

With reference to the number of plants obtained from hulled and nonhulled seed, the data of table 1 give further support to previous investigations (9) in showing that, in general, seed hulling results in a reduced stand.

DISCUSSION

From the data presented, it is evident that the removal of hulls from naturally inoculated seed oats involves factors in addition to reduction in inoculum load that the hulls may carry. Considering the nature of the tightly appressed hulls, it seems possible that they may serve also as buffers in protecting the inoculum in the pericarp against dessication, flooding, sudden changes in temperature, possible antagonistic organisms and other soil factors that inhibit or prevent infection. As noted, the apparent influence of the hulls in this respect varied widely under different environmental conditions. Doubtless the possible interplay of different soil factors covers a wide range of conditions that might variously influence both the protective action of the hulls and the physiologic relations of the various combinations of host varieties and physiologic forms of the pathogen in the different seed lots.

In a consideration of methods of artificially inoculating seed oats with loose smut, it seems evident, therefore, that the position of inoculum with reference to protection by the hulls may prove to be an important factor in the approach to results comparable to those in nature. This protection is not obtained by coating the surface of nonhulled seed with dry smut spores. Furthermore, as shown by Johnston (6), this method also may enable susceptible varieties to escape infection through the interference of the hulls. Application of dry spores to hulled seed eliminates the latter factor and generally results in high percentages of smut, as shown by Stanton *et al.* (9). Hulling, however, is a slow and tedious operation. Zade and Arland (13) report that it required 32 8-hour days to hull and inoculate one kilogram (2.2 lbs.) of oats. These investigators also observed that under natural conditions and with the "evacuation" method of inoculation, plants from the inner seeds of the spikelets are more susceptible than those from the outer seeds. The application of a heavy spore load to the hulled seed, however, resulted in an attack so severe as to obliterate this natural difference. A further complication is introduced by the fact shown by Stanton *et al.* (9) that hulling not only reduced seedling emergence but the extent of reduction varies considerably in different varieties. The writer's recent observa-

tions on different lots of oats have shown a notable difference in the susceptibility of nonhulled and hulled seed to mold attack. The shrivelled, hulled seeds attached to week-old seedlings dug from the soil, were thickly covered with blue, black, and pink molds. The comparable nonhulled seeds were bright and macroscopically mold-free. In view of this fact, the mold attack rather than injury from the process of hulling may be chiefly responsible for the reductions in seedling emergence that usually accompanies hulling. As shown by Stanton *et al.* (9) these reductions occur even when the seed is not inoculated with smut. The likely influence of this unnatural prevalence of mold on the smut inoculum of hulled seed doubtless raises a further question as to the desirability of using hulled oats.

In the light of the results here recorded it should be noted, moreover, that the method of applying spores to hulled seed fails to include not only the influence of the protection of inoculum by the hulls but also the variable extent of this protection occasioned by the fact that the length, thickness, and adhesiveness of the hulls varies in different varieties.

The "evacuation" inoculation method described by Zade (12) and Haarring (4, 5) apparently is more satisfactory in expediting results comparable to those in nature. In this method, seed is placed in a spore suspension and the spores are forced beneath the hulls by the release of a vacuum. The moist seed is then stored for 20 hours to promote spore germination and the spread of subsurface inoculum. This method, however, would be difficult to apply in physiologic-form studies, as it is necessary to inoculate many small lots of seed with different collections of smut. Doubtless further experimentation would be desirable to determine the comparability of results from various artificial methods of seed inoculation and from the natural method, under widely different conditions of seeding.

SUMMARY AND CONCLUSIONS

In the usual artificial methods of inoculating seed oats with loose smut, dry spores are applied to the surface of nonhulled or hulled seed. The inoculum, therefore, comes into direct contact with the soil, whereas the effective inoculum in naturally inoculated seed resides in the superficial seed structures enclosed and protected by the siliceous layers of the hulls.

To determine the significance of the protection afforded by the hulls in seedling infection and on subsequent smut in oats, nonhulled and hulled seed of 10 seed lots, naturally inoculated with loose smut, was sown in the field and greenhouse at Rosslyn, Va. (Arlington Experiment Farm). Seven of the lots also were sown in the field at Aberdeen, Idaho, and grown under irrigation. This procedure also permitted a further study of the moot question whether inoculum in the hulls or pericarp is accountable for most of the

seedling invasion and loose smut in oats grown from naturally inoculated seed.

The results showed that hulling is not a reliable basis on which to determine whether inoculum in the hulls or pericarp is accountable for most of the loose smut in oats. The different conditions in the 3 locations of the experiment so markedly influenced the range of reduction in smut due to hulling that inoculum either in the hulls or pericarp could be held accountable for most of the smut. Within individual lots the percentages of reduction in smut in plants grown from hulled seed varied as widely as from 2.1 to 90.2 in the 3 locations of the experiment. In the different lots grown under similar conditions wide variations in smut reduction from hulling also occurred.

In general, relatively unfavorable conditions for smut were more acutely reflected in plants from hulled seed than in plants from nonhulled seed.

The inconsistent reductions in smut resulting from hulling indicate that hulling involves factors in addition to reduction in the load of inoculum carried by the hulls. Apparently the tightly adherent hulls also may serve more or less effectively as buffers in protecting inoculum in the pericarp against various soil influences inimical to infection. In the present experiments the extent of this influence varied widely under different conditions.

Hulled seed of all of the 10 seed lots produced some smutted plants; evidence that at least a portion of the loose-smut inoculum resided in the caryopses of these lots.

In a discussion of the various artificial methods of inoculating seed oats with loose smut, it is noted that present methods are laborious and/or not applicable to all types of investigation and/or that they fail to involve the influence of the seed hulls and introduce unnatural and variable factors that may mask the true status of resistance or susceptibility as it occurs in nature. Further experimentation is desirable to determine the comparability of results from various artificial methods of seed inoculation and from the natural method, under widely different conditions of seeding.

LITERATURE CITED

1. ARLAND, A. Der Hafer-Flugbrand, *Ustilago avenae* (Pers.) Jens. Biologische Untersuchungen mit besonderer Berücksichtigung der Infektions- und Anfälligkeitsfrage. Bot. Arch. **7**: 70–111. 1924.
2. DIEHL, O. Experimentelle Untersuchungen über die Lebensweise und Bekämpfung des Haferflugbrandes. Bot. Arch. **11**: 146–199. 1925.
3. GAGE, G. R. Studies on the life history of *Ustilago avenae* (Pers.) Jensen and of *Ustilago levis* (Kell. and Swing.) Magn. Cornell Agr. Expt. Sta. Mem. 109. 1927.
4. HAARRING, F. Eine Infektionsmethode für Haferflugbrand (*Ustilago avenae* Jens.)

und ihre Anwendung zu Beiz- und Immunitätsversuchen im Laboratorium und Feld. Bot. Arch. **29**: 444–473. 1930.

5. HAARRING, F. Beizmittelprüfung an Haferflugbrand im Laboratorium mit der "Leipziger Methode." Pflanzenbau **7**: 89–92. 1930.
6. JOHNSTON, C. O. Effects of soil moisture and temperature and of dehulling on the infection of oats by loose and covered smuts. Phytopath. **17**: 31–36. 1927.
7. ROESCH, A. Studien über den Haferflugbrand, *Ustilago avenae* (Pers.) Jens. und den Glatthaferbrand, *Ustilago perennans* Rostr., mit besonderer Berücksichtigung der Immunitätsfrage beim Haferflugbrand. Bot. Arch. **13**: 382–432. 1926.
8. SAMPSON, K. The biology of oat smuts. II. Varietal resistance. Ann. Appl. Biol. **16**: 65–85. 1929.
9. STANTON, T. R., F. A. COFFMAN, V. F. TAPKE, G. A. WIEBE, R. W. SMITH, and B. B. BAYLES. Influence of hulling the caryopsis on covered-smut infection and related phenomena in oats. Jour. Agr. Res. [U. S.] **41**: 621–633. 1930.
10. ZADE, A. Experimentelle Untersuchungen über die Infektion des Hafers durch den Haferflugbrand (*Ustilago avenae* (Jens.). Fühling's Landw. Ztg. **71**: 393–406. 1922.
11. ————————. Neuere Untersuchungen über die Lebensweise und Bekämpfung des Haferflugbrandes (*Ustilago avenae* (Pers.) Jens.). Angew. Bot. **6**: 113–125. 1924.
12. ————————. Masseninfektionen mit Haferflugbrand nach einem neuen Verfahren. Pflanzenbau **5**: 43. 1928.
13. ————————, and A. ARLAND. The relation of host and pathogen in *Ustilago avenae:* a reply. Bull. Torrey Bot. Club **60**: 77–87. 1933.

COMPARATIVE STUDIES OF SOME EUROPEAN AND AMERICAN POTATO VIROSES[1]

T. P. DYKSTRA

(Accepted for publication July 12, 1935)

Viroses affecting potatoes have been described in Europe and America. Since the potato varieties in Europe differ from those in America and the symptoms of potato viroses may vary considerably, depending upon the variety, it is impossible to determine the identity of the viruses found on the two continents unless the diseases caused by them are studied on the same varieties under similar conditions.

Until the identity of the viroses affecting the potato in Europe and America is determined, it is not possible to apply the extensive knowledge gained in one continent to the solution of related problems of the other.

In order to study foreign and American viroses on European as well as American varieties, several virus-diseased and healthy tubers of different varieties were secured from England through the courtesy of Dr. R. N. Salaman. Since healthy tubers of European varieties are generally free from the X-virus (latent mosaic universally present in so-called "healthy" commercial American varieties), it was felt desirable for comparison of the viroses to remove the X-component from the American virus complexes. This was readily accomplished by grafting scions of virus-diseased potatoes to the U.S.D.A. Seedling No. 41956, which is resistant to this latent virus (8). Tubers of this seedling were kindly supplied by E. S. Schultz, of the Bureau of Plant Industry, United States Department of Agriculture.

By this procedure, it was possible to secure leaf-rolling mosaic, crinkle mosaic, mild mosaic, and rugose mosaic free from the X component. These virus components, with the possible exception of leaf-rolling mosaic also were secured free from the X virus by transferring the diseases to tobacco by means of aphids, which do not transfer the latent potato mosaic.

The European viroses have been studied in comparison with American material under the same conditions on different American and European varieties, and in many cases on plants that came from the same tuber unit. Although the work is not nearly completed, sufficient progress has been made to warrant drawing certain conclusions. It is believed opportune to present this preliminary note.

The studies were started in the greenhouse at Corvallis, Oregon, in the

[1] This paper is based upon investigations carried on as a cooperative project between the Division of Fruit and Vegetable Crops and Diseases, Bureau of Plant Industry, U. S. Dept. of Agriculture, and the Oregon Experiment Station at Corvallis, Oregon.

fall of 1933. It was found that, with the exception of the Y virus, the grafting method of transferring the European viroses was far more successful than the leaf-mutilation method. Stem grafts of diseased scions were made to healthy potted plants, which were then kept in a moist chamber for about 10 days. Then they were removed and kept in the greenhouse for further observations, according to the method recommended by Salaman and Le Pelley (7). When this procedure was followed, about 80 per cent of the grafts were successful.

After the plants had matured, the tubers were harvested, stored in paper bags, and planted the following spring under muslin-covered insect-proof cages in the field. This procedure makes it possible to secure current-season, and tuber-perpetuated symptoms within a year.

The general conclusions reached are summarized in the following paragraphs:

Y Virus and Vein-banding Virus. Smith (9) refers to potato virus Y as one of the commonest and most destructive of potato viruses in England. He states that it is common in American tobacco fields, where it is known as the vein-banding virus. The American vein-banding virus is the aphid-transmitted component of rugose mosaic. Koch (1). This virus has been studied by the writer in comparison with the Y virus on tobacco and on different American and European potato varieties.

Both of these viroses produce on tobacco a banding of the veins, but the symptoms produced by the Y virus are very striking and distinct, whereas those produced by the vein-banding virus are very mild in comparison. Both of these viroses in conjunction with virus X produce spot necrosis on tobacco.

The current-season symptoms of the Y virus on Irish Cobbler, White Rose, Burbank, Bliss Triumph, Green Mountain, and Earliest of All, which also harbor X virus, are manifested by necrotic leaf spots including the veins and the tissue immediately surrounding them. Nearly every leaf on the plant may eventually drop, and remain hanging on the main stem by a thread of tissue, leaving only a tuft of leaves at the tip of the plant.

Plants from tubers of plants inoculated with Y virus appear at first as if infected with rugose mosaic, except that the necrosis of the veins instead of being confined to the lower leaves, is found on almost every leaf. While the plant is still immature, the lower leaves, at first green, gradually turn yellow and drop, but remain hanging on the stem by a thread of tissue (fig. 1).

In the variety Bliss Triumph the petioles develop necrotic spots and stripes of streak are found on the stem. Finally, all of the lower leaves dry down and hang on the main stem. At this stage of development the disease appears to be quite different from rugose mosaic.

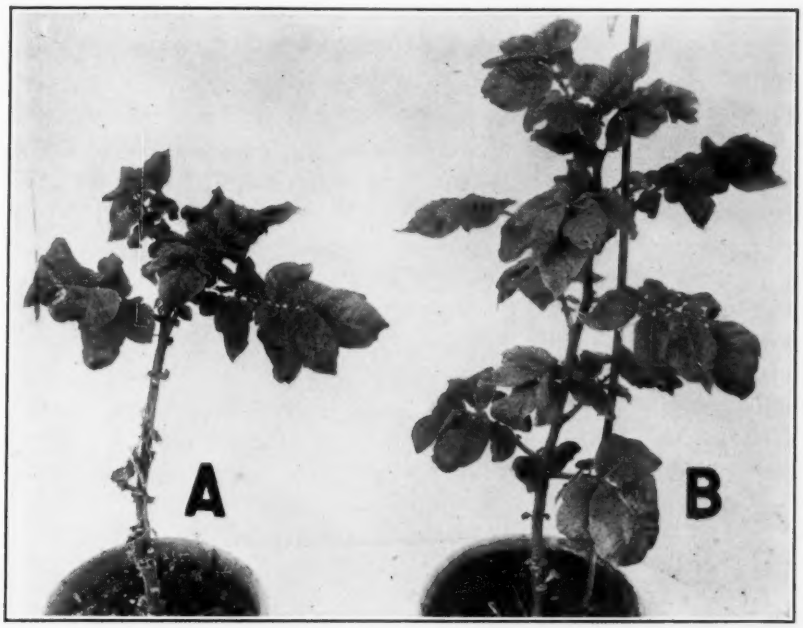


FIG. 1. Tuber-perpetuated symptoms of viroses on Bliss Triumph in the greenhouse. A. Y virus necrosis of the veins is evident on practically every leaf. Note the severe leaf drop. B. Vein-banding virus. Necrosis of the veins is evident on the lower leaves only. Note the absence of leaf drop.

When the vein-banding virus is inoculated into potato plants containing the X virus, current-season symptoms of rugose mosaic develop. It was found that under greenhouse conditions the leaves did not always develop a necrotic spotting, as a result of inoculation of the above-named varieties with the vein-banding virus. In some cases a diffused mottling, typical of rugose mosaic, developed without any noticeable necrosis.

Plants from tubers of plants inoculated with vein-banding virus showed typical rugose-mosaic symptoms, evidenced by a rugosity of all the leaves and a necrosis of the veins of the lower leaves. In no case was any leaf dropping observed on tuber-perpetuated rugose mosaic plants of the different varieties tested. In these cases, necrosis was not evident in the petioles, nor was streaking of the stem observed in infected plants of the variety Bliss Triumph.

In the English variety President, the symptoms of the second generation of the Y virus manifest considerable leaf drop and considerable necrosis of the veins in addition to crinkling and diffused mottling of the leaves.

The tuber-perpetuated symptoms of the vein-banding virus on President differ from the Y virus in that it fails to cause an appreciable necrosis of

the veins or leaf drop, but results only in a diffused mottling of the leaves. The symptoms of these two viroses in this variety have been observed on more than 100 plants.

Both of these viroses cause on Arran Victory diffused mottling without any necrosis or leaf drop. The vein-banding virus causes only a diffused mottling on Epicure, whereas the Y virus caused a diffused mottling and a slight necrosis of the veins of the lower leaves.

In order to determine whether or not the Y virus has any effect upon potato plants infected with rugose mosaic the following experiment was started in the spring of 1935. Each of 6 tubers of the variety Green Mountain and 6 tubers of Bliss Triumph infected with rugose mosaic were cut into 3 pieces. Each seed piece was planted in a 6-in. pot in the greenhouse. When the plants were about 3 or 4 inches high, those derived from 1 seedpiece of each tuber were inoculated with the Y virus by means of the rubbing method. Plants from another seedpiece of each tuber were inoculated with the vein-banding virus, and the other seedpieces were not inoculated and served as controls.

These plants were kept under observation for more than 2 months after inoculation, but none of the plants developed symptoms different from the controls, which showed the typical tuber-perpetuated rugose-mosaic symptoms.

It appeared as if the rugose-mosaic virus had an inhibiting effect upon the expression of the Y virus and prevented the development of the typical Y-virus symptoms. The tubers from the inoculated plants were saved and will be planted later to determine whether the introduction of the Y virus or additional vein-banding virus into rugose-mosaic-infected potato plants has any effect on the symptoms in the progeny.

Further studies with these 2 viroses are in progress, but the results secured to date have proven conclusively that they belong to the vein-banding group but are not identical. The effect of the Y virus on potato plants is more severe than that of the vein-banding virus.

Paracrinkle. This disease was first described by Salaman and Le Pelley (7). Murphy and McKay (2) have suggested that this virus may have affinities with leaf-rolling mosaic. We have studied this virus in comparison with leaf-rolling mosaic on the American varieties, Burbank, White Rose, Earliest of All, and Bliss Triumph, and failed to find any resemblance in the symptoms of these two diseases. Instead of diffused mottling and a rolling of the leaves, which are the characteristic symptoms of leaf-rolling mosaic, paracrinkle developed large mosaic-like blotches on the foliage, and failed to produce any rolling of the leaves.

Leaf-rolling mosaic, free from X virus, as well as the complex leaf-rolling

mosaic and the X virus, have been transferred to Arran Victory. The symptoms of this disease on this variety are characterized by a diffused mottling and rolling of the leaves in contrast to the blotchy mottling and puckering of the leaves caused by paracrinkle.

In the variety Burbank, pin-point-like necrotic spots developed on the leaves in addition to the mottling when infected tuber-perpetuated plants were grown under cages in the field. (Fig. 2.) These necrotic spots, how-

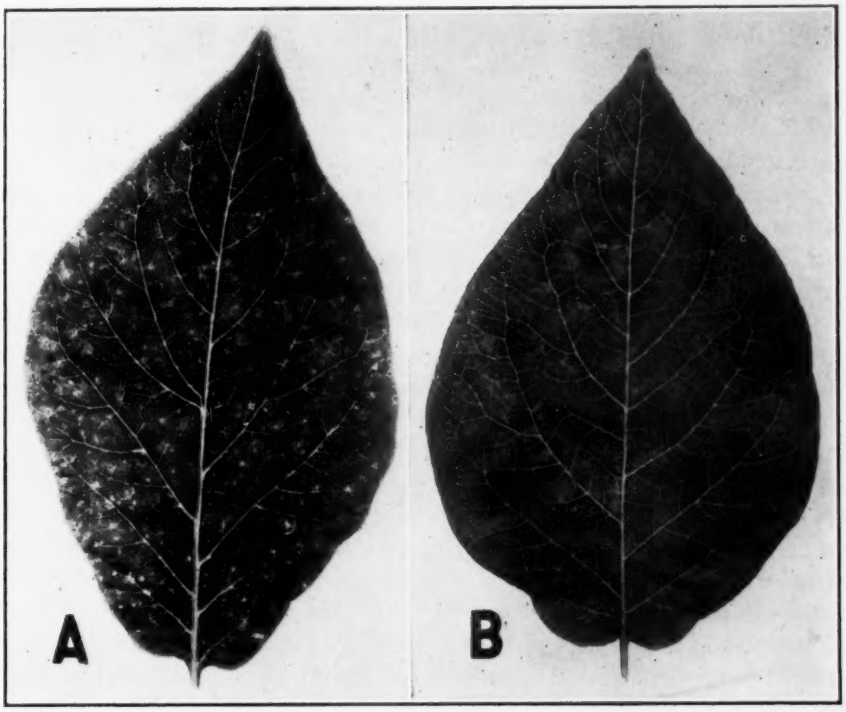


FIG. 2. Tuber-perpetuated paracrinkle symptoms on Bu.bank. A. On plants growing under cages in the field. Note pin-point-like necrotic spots on leaves. On the print they resemble thrips injury, but these insects were not present in the cage. B. In the greenhouse. Note the large irregularly shaped mosaic-like blotches.

ever, were not evident on the progeny from these plants when planted in the greenhouse. Paracrinkle appears to be entirely different from any of our American diseases.

Crinkle A. This disease is well known in Europe and has been described as crinkle by Murphy, Quanjer, and Salaman (2, 3, 4, 6). The last of whom refers to it as crinkle A to distinguish it from paracrinkle.

It is composed of at least one virus component in addition to X. That

the X virus is the same as the American latent mosaic (present in commercial "healthy" potatoes) was indicated when juice transfer of this virus to Jimson weed resulted in the characteristic mottling of the leaves. By grafting scions of infected President to seedling No. 41956 (resistant to the latent virus), the X virus was eliminated and the other component was retained. This component has been studied on tobacco plants in comparison with the viruses from American mild mosaic and crinkle mosaic from which the latent mosaic component had been removed. There is no observable consistent difference in the symptoms produced by these three viroses on tobacco plants. Instead of vein-banding, a mottling develops which resembles the mild mottling sometimes produced by the X virus. It was found that it can be readily distinguished from this virus, however, by inoculating infected plants with tobacco mosaic. The tobacco plants infected with the X virus upon addition of tobacco mosaic will invariably develop a spot necrosis on the leaves, whereas the plants infected with the other viruses will develop only tobacco-mosaic symptoms in addition to the mottling due to the particular potato-mosaic virus present.

These viruses are readily transmitted to tobacco, and 100 per cent infection was invariably secured when a small amount of carborundum dust was added to the extracted juice from the leaves of an infected plant, and a piece of absorbent cotton rolled into the end of a swab stick was dipped into the juice and rubbed lightly on the tobacco leaves (5).

These three viroses, namely, mild mosaic, crinkle mosaic, and Crinkle A, free from the latent virus, have been transferred from tobacco to Bliss Triumph potato plants by juice transfers and also by grafting, and have resulted in the development of typical symptoms characteristic of each disease in this variety.

Studies on the identity of crinkle A are not yet completed, but the results secured indicate its resemblance to those of mild mosaic, and crinkle mosaic, yet it differs from them. The most striking difference in symptoms among these three viroses was found on the X resistant seedling. On this host the crinkle-A virus fails to produce any appreciable leaf crinkle, and, while the mottling is more uniformly spread over the leaf, the mottled areas are not so chlorotic as those produced by the other two viroses (Fig. 3). Crinkle mosaic resembles mild mosaic on this host, except that the former causes considerably more crinkling of the foliage. These three diseases also have been studied on Bliss Triumph, Green Mountain, Earliest of All, and Burbank, and, although there is a difference in symptoms produced on these varieties, it is not so pronounced as in the seedling. Crinkle A resembled mild mosaic more than crinkle mosaic. Of three diseases, crinkle A produced the least crinkling of the leaves.

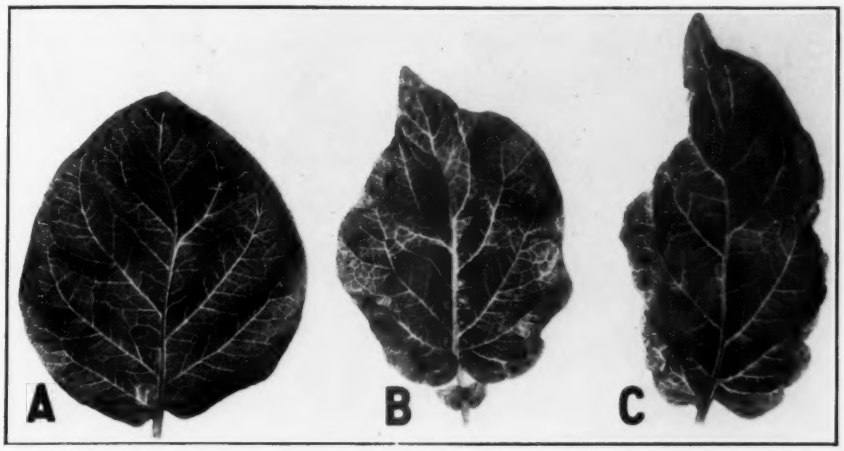


FIG. 3. Leaflets of X resistant U. S. D. A. seedling No. 41956 showing tuber-perpetuated symptoms of the following diseases: A. Crinkle A. B. Mild-mosaic. C. Crinkle mosaic. Note the variation in types of mottling and degrees of crinkling produced in these three different mosaics.

Murphy and McKay (2) also state that crinkle A is closely related to the American mild mosaic.

Top necrosis Di Vernon. This virus has been described by Bowden (according to Smith) (10, p. 317) who refers to the component causing top necrosis in certain potato varieties as virus C. In addition to this the X component also is present.

In the fall of 1933 a few grafts of scions from infected Di Vernon plants were made to some American potato varieties, but current season symptoms failed to develop. When the progeny from these plants were planted under cages, only two plants from one hill lot of Bliss Triumph developed symptoms, which were characterized by extreme dwarfing of the plant and a necrosis of the veins of most of the leaves.

In the fall of 1934 several plants of the variety Burbank, Bliss Triumph, Irish Cobbler, and Earliest of All were grafted with scions from infected Di Vernon plants. In nearly all cases current-season symptoms developed that were characterized in these varieties by a severe top necrosis, consisting of a streaking of the stem and petioles, and by numerous small circular necrotic spots on the foliage (fig. 4).

The second generation symptoms of this disease on these varieties as observed on plants growing under cages in 1935, were manifested by a slight mottling of the foliage, without any noticeable dwarfing and in many cases without any indications of necrosis. In a few plants of Irish Cobbler circular necrotic spots were found on the lower leaves.

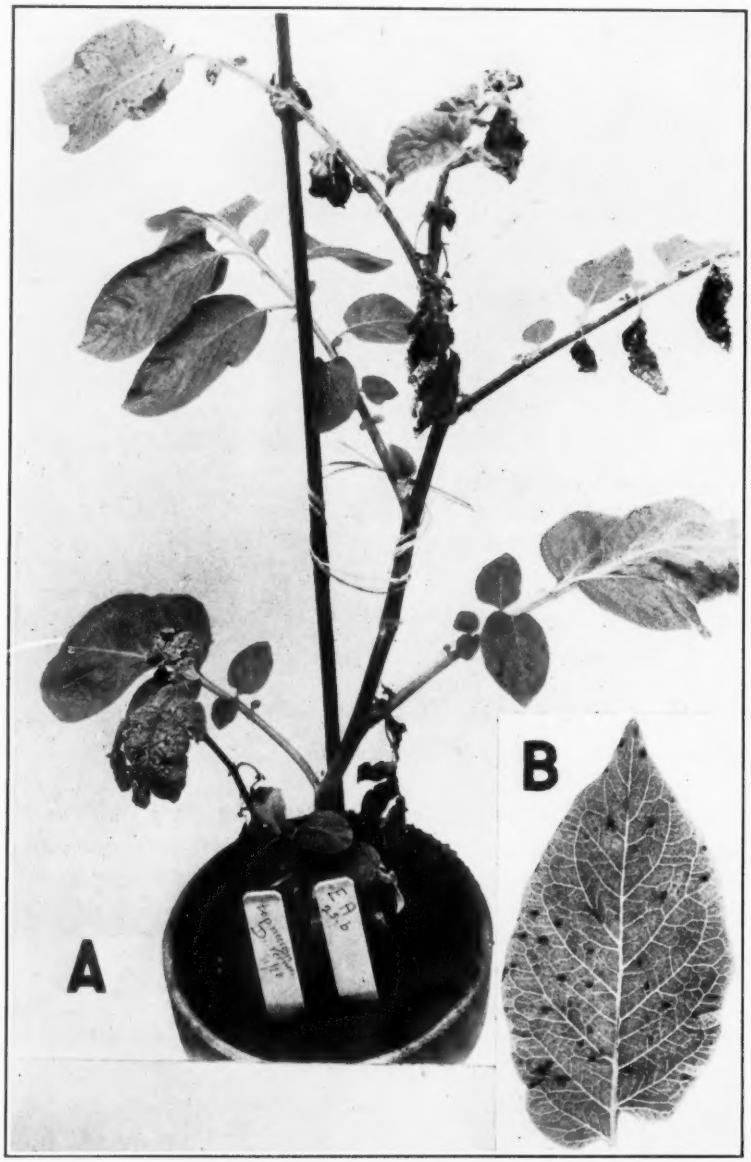


FIG. 4. Current-season symptoms of top necrosis produced by virus C. A. On Earliest of All. Note necrotic spotting on the foliage and petiole. B. On Irish Cobbler. Note necrotic spots.

The fact that in the one positive case of 1934 current-season symptoms had failed to develop, may account for the severe effect of this disease in the second generation. The writer has observed that in some cases when rugose-mosaic infection took place late in the season and current-season symptoms failed to develop, the second generation symptoms the following year closely resembled current-season infection.

The X component has been removed from this virus complex by grafting scions from plants affected with top necrosis Di Vernon to resistant seedling No. 41956. This C virus has successfully been transferred from this seedling to President and Majestic. The effect of this virus on plants free from the X virus has not yet been extensively studied.

Virus C does not correspond to any of the known potato viruses found in America.

SUMMARY

Comparisons of some European and American potato viroses were made on a number of potato varieties from both continents. The X virus was found to be similar to the so-called latent virus of "healthy" American commercial potatoes. The Y virus, and the vein-banding virus (rugose mosaic complex from which the X component has been removed) produce a banding of the veins on tobacco leaves. These two viroses belong to the same group but are not identical. Whereas the tuber-perpetuated vein-banding symptoms are manifested on most American varieties containing the X virus by a necrosis of the veins of the lower leaves, without leaf drop, American varieties infected with tuber-perpetuated Y virus showed not only a necrosis of the veins on practically every leaf of the plant but also considerable leaf drop. This virus caused a necrosis of the petioles and streaking in the stem of Bliss Triumph.

Paracrinkle does not resemble leaf-rolling mosaic or any other known American potato virosis. It produces large, irregular-shape mosaic-like blotches on the foliage of American varieties. Under cages, in the field, pin-point-like necrotic spots, in addition to a mottling, developed in the leaves of infected Burbank plants.

Crinkle A is not identical with rugose mosaic. It resembles mild mosaic, but the mottled pattern is somewhat different and the crinkling of the leaves is less severe than that caused by mild mosaic on the same varieties.

Virus C produced a severe top necrosis as current season symptoms on every one of the American potato varieties tested. This virus does not resemble any of the known viruses affecting potatoes in this country.

OREGON STATE COLLEGE,
CORVALLIS, OREGON.

LITERATURE CITED

1. KOCH, KARL. The potato rugose mosaic complex. Science, n. s., **73**: 615. 1931.
2. MURPHY, PAUL A., and R. MCKAY. A comparison of some European and American virus diseases of the potato. Roy. Dublin Soc. Sci. Proc., n. s., **20**: 347–358. 1932.
3. MURPHY, PAUL A. Investigation of potato diseases. Canada Expt. Farms Bul., 2d ser., 44. 1921.
4. QUANJER, H. M. General remarks on potato diseases of the curl type. Internat. Conference of Phytopath. and Econ. Ent., Holland, Report **1923**: 23–28.
5. RAWLINS, T. E., and C. M. TOMPKINS. The use of carborundum as an abrasive in plant-virus inoculations. (Abst.) Phytopath. **24**: 1147. 1934.
6. SALAMAN, R. N. Crinkle "A," an infectious disease of the potato. Roy. Soc. [London] Proc., Ser. B., **106**: 50–83. 1930.
7. ————————, and R. H. LE PELLEY. Para-crinkle: a potato disease of the virus group. Roy. Soc. [London] Proc., Ser. B., **106**: 140–175. 1930.
8. SCHULTZ, E. S., and W. P. RALEIGH. Resistance of potato to latent mosaic. (Abst.) Phytopath. **23**: 32. 1933.
9. SMITH, K. M. On the composite nature of certain potato virus diseases of the mosaic group. Roy. Soc. [London] Proc., Ser. B., **109**: 251–267. 1931.
10. ————————. Recent advances in the study of plant viruses. 423 pp. P. Blakiston's Son and Co., Philadelphia. 1933.

PHYTOPATHOLOGICAL NOTES

An Apparent Natural Transfer of the Bulb or Stem Nematode from Clover to the Strawberry Plant.—An instance of what appears to be a natural transfer of the bulb or stem nematode, *Anguillulina dipsaci*, from red clover to Marshall strawberries has been observed in the Pacific Northwest.

The strawberry plants were grown on part of a field formerly in red clover that had a known infestation by the bulb or stem nematode for the last several years. During 1933 the farmer plowed a portion of this field and planted the newly plowed area to Marshall strawberries (Fig. 1). In

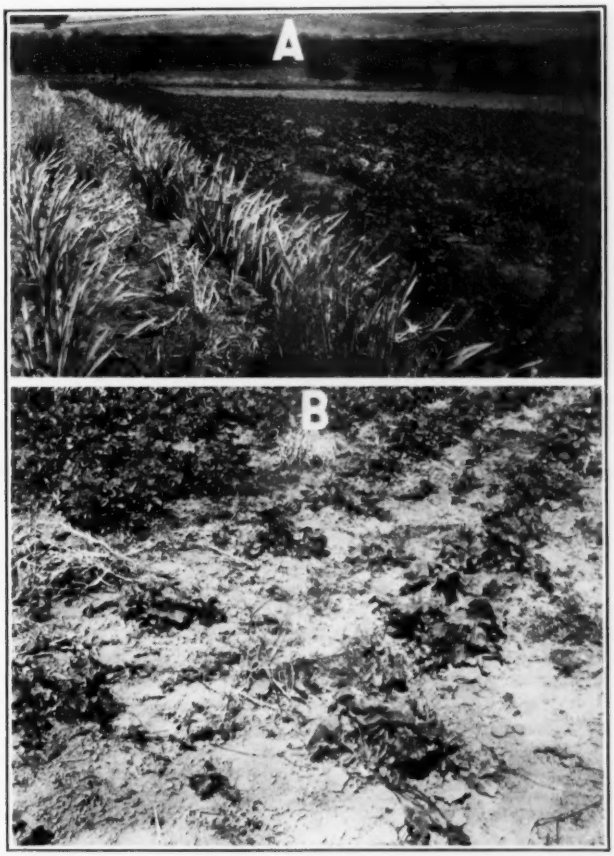


FIG. 1. A. General view of strawberry field showing two rows of uninfested narcissus plants on left. Blank area at back is location where strawberry runners failed to grow, due to *A. dipsaci*. B. Closeup of infested area in strawberry field shown at left. Note irregular stand and distorted plant in foreground.

the early spring of 1935, 4 acres more of the infested field were plowed and planted with runners from the above mentioned strawberry plants. When these young plants failed to grow properly, the farmer called his county horticultural inspector who submitted clover plants from the original clover field and the mother strawberry plants and runners to the present writer for

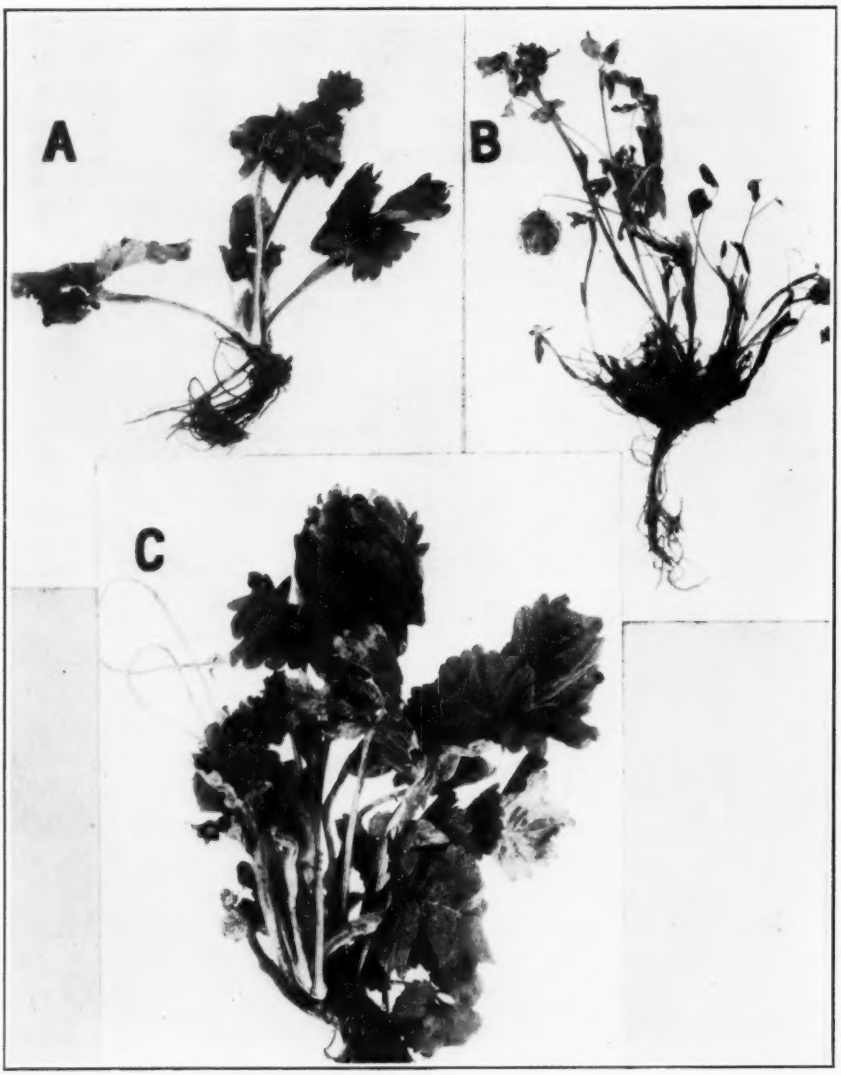


FIG. 2. A. Runner showing swollen stems and leaf distortion caused by *A. dipsaci*. B. Clover plant nearly killed by infestation of *A. dipsaci*. C. Mother plant showing same symptoms as runner.

an examination as to the presence of a nemic disease. All these plants exhibited marked nemic-disease symptoms (Fig. 2), and upon microscopic examination, showed large numbers of *Anguillulina dipsaci* in all stages of development.

From the following circumstantial evidence it seems probable that in the present case the red-clover population of *Anguillulina dipsaci* has transferred, under natural conditions, to Marshall strawberries:

1. A check of the source of supply of the mother strawberry plants failed to show any nematode infestation or history.
2. Other strawberry growers, who secured their planting stock from the same place at the same time as did the grower concerned herein, have no nemic infestation in their fields.
3. The field on which the strawberry plants were grown had never before been planted to strawberries.

Infested plant material has been secured from these fields, so that experimental evidence also may be furnished.

In view of the importance of the host specificity problem in the study of plant parasitic nematodes and their control, observations like the present one are of so much value that they should be recorded.—WILBUR D. COURTNEY, Sumner, Washington.

Greenhouse Wires and Pipes Galvanized with Zinc React with Sulphur Dioxide to Form Soluble Zinc Salts.—In the summer of 1935 sulphur dioxide fumigation, along with formaldehyde soil sterilization, was recommended to help clean up several greenhouse diseases, but later in the fall severe injury occurred to a crop of tomatoes. The outbreak of the injury always was correlated with the dripping of condensed moisture or rains from wires and pipes. Yellowish white to white crystals were observed on all zinc-galvanized surfaces. In the presence of water these crystals dissolved slowly and the solution dripped on the plants below. Wherever the solution touched the tomato plants, black to blackish brown lesions were produced. In many cases the plants were severely injured. This same injury was reported in 1933 by Read and Orchard[1] on chrysanthemums under somewhat similar conditions in England. These workers observed that zinc sulphate was formed on galvanized wires and pipes as well as surfaces that were painted with paints containing "zinc white" (zinc oxide) as their base, if sulphur was burned in a humid atmosphere.

The problem in Illinois deals with wires and pipes galvanized with metallic zinc. If fumigation is done in a humid atmosphere the moist sul-

[1] Read, W. H., and Orchard, O. B. Plant injury following the burning of sulfur in glasshouses. Experimental and Research Station, Turner's Hill, Cheshunt, Herts. Ann. Rpt. 19: 98–108, 1933 (4).

phur dioxide can be expected to react with the zinc on the pipes, wherever moisture has condensed, to form zinc thiosulphate,[2] a soluble greenish yellow solid with the formula ZnS_2O_3. Under greenhouse conditions it is very probable that the zinc thiosulphate would oxidize to zinc sulphate. Both of the salts are soluble in water and capable of injuring plants if a too concentrated solution touches them.—K. J. KADOW, W. A. RUTH and H. W. ANDERSON, University of Illinois, Urbana, Illinois.

[2] Roscoe, H. E., and Schorlemmer, C. Treatise on Chemistry 2: 6th Edition, 1923. MacMillan.

PHYTOPATHOLOGICAL SUMMER MEETING

IOWA STATE COLLEGE, AMES, IOWA
JUNE 25–26, INCLUSIVE

First Day

9:00 A.M.: Registration, Botany Hall, Room 202.
Tour through Laboratory and Greenhouses.

1:00 P.M.: Tour of Phytopathological Plots, Agronomy Farm, Horticultural Orchards, and Federal Erosion Nursery.

6:30 P.M.: Dinner, Memorial Union.

8:00 P.M.: Extension Workers' Round-table Discussion.
Coordination of Programs.

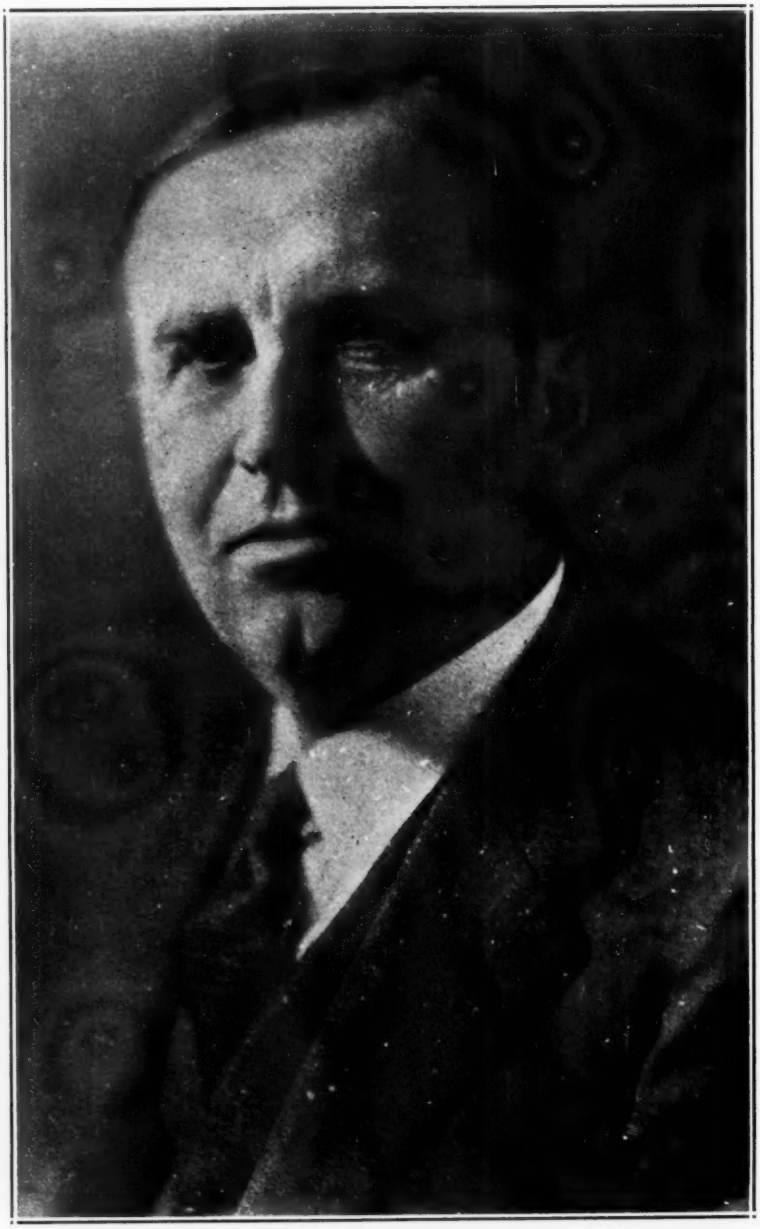
9:00 P.M.: Round-table Discussion—Research.
Relation of Phytopathology to Erosion Control.

Second Day

8:00–10:30 A.M.: Research—Round-table Discussion.
Is There a Place for Regional Research Programs in Plant Pathology?

12:30 P.M.: Dinner, Leader Cafe, Kanawha.

1:30 P.M.: Excursion to Northern Iowa Experimental Farm, Kanawha, Iowa—Cereals, Beets, Potatoes, etc.
Excursion to Southeastern Experimental Farm, Conesville, Iowa—Melons, Sweet Potatoes, Other Truck Crops.
Excursion to Western Iowa Fruit Section, Glenwood, Iowa—Apple Spraying, Apple Root Rot, Nursery Diseases.



JAMES M. VAN HOOK